FUMARATE-BASED POLYMERS: OLIGO(POLY(ETHYLENE GLYCOL) FUMARATE) AND POLY(BUTYLENE FUMARATE) FOR TISSUE ENGINEERING HEART VALVE AND BONE TISSUE

Christian T. Denny
University of New Mexico - Main Campus

Follow this and additional works at: https://digitalrepository.unm.edu/bme_etds

Part of the Molecular, Cellular, and Tissue Engineering Commons, and the Other Medicine and Health Sciences Commons

Recommended Citation
Denny, Christian T. "FUMARATE-BASED POLYMERS: OLIGO(POLY(ETHYLENE GLYCOL) FUMARATE) AND POLY(BUTYLENE FUMARATE) FOR TISSUE ENGINEERING HEART VALVE AND BONE TISSUE." (2022). https://digitalrepository.unm.edu/bme_etds/34

This Dissertation is brought to you for free and open access by the Engineering ETDs at UNM Digital Repository. It has been accepted for inclusion in Biomedical Engineering ETDs by an authorized administrator of UNM Digital Repository. For more information, please contact disc@unm.edu.
Christian Tyler Denny

Candidate

Biomedical Engineering

Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Elizabeth L. Hedberg-Dirk, PhD., Chairperson

Christina Salas, PhD.

Andrew P. Shreve, PhD.

Nick J. Carroll, PhD.
FUMARATE-BASED POLYMERS: OLIGO(POLY(ETHYLENE GLYCOL) FUMARATE) AND POLY(BUTYLENE FUMARATE) FOR TISSUE ENGINEERING HEART VALVE AND BONE TISSUE

by

CHRISTIAN T. DENNY

Degrees:

B.S. Biology, 2015
Idaho State University, Pocatello, ID

M.S. Biomedical Engineering, 2017
University of New Mexico, Albuquerque, NM

DISSERTATION

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Engineering

The University of New Mexico
Albuquerque, New Mexico

May 2022
Acknowledgments

I would like to thank my advisor Elizabeth Dirk for educating and guiding me through the Ph.D. process. I am also grateful for the mentorship and training of my other advisors, namely: Dr. Christina Salas, Dr. Andy Shreve, and Dr. Nick Carroll. My education would not have been possible during such unusual circumstances without their support. I want to also thank Dr. Matt Rush for his consistent guidance and willingness to help me through my Ph.D. Thank you to the numerous students and mentees I have taught and learned from, in turn: Dr. Kent Coombs, Quan, Derek, Blaise, Diego, Jasmine, Dr. Zhen Zhong, and many more. Without their assistance, I would have never been able to finish my Ph.D.

Thank you to my family, especially my parents Byron and Louisa, for your love and support throughout my academic experience. You have always encouraged me to complete my goal of obtaining an advanced degree. I also want to thank my grandparents Gene and Martha who have always cheered for me through this long journey but never got to see me finish. Finally, I want to thank my wife, Holly. Your unwavering patience, support, and sacrifice have allowed me to obtain this degree over the many years. Here is to the next big adventure in life.
FUMARATE-BASED POLYMERS: OLIGO(POLY(ETHYLENE GLYCOL) FUMARATE) AND POLY(BUTYLENE FUMARATE) FOR TISSUE ENGINEERING HEART VALVE AND BONE TISSUE

By: Christian Tyler Denny

B.S. Biology, 2015
Idaho State University, Pocatello, ID

M.S. Biomedical Engineering, 2017
The University of New Mexico, Albuquerque, NM

Ph.D. Engineering, 2022
The University of New Mexico, Albuquerque, NM

ABSTRACT

Tissue engineering is an emerging field that came from the fields of medicine, materials science, and engineering. The foundation of tissue engineering uses a paradigm that incorporates cells, biomaterials, and exogenous factors to create living tissues for medical, pharmaceutical, and research purposes. Within the last 100 years, biomaterials have been developed to solve many medical and research problems. As biomaterials have developed throughout the years new materials have been developed to have specific properties appropriate for medical applications. Fumarate is a naturally derived molecule in the body and has been found useful for developing polyesters that can be crosslinked into degradable biomaterials. This work explores a new method for synthesizing oligo(poly(ethylene glycol) fumarate) (OPF) and poly(butylene fumarate) (PBF). OPF was used to create a positively charged hydrogel platform for aortic valve engineering and PBF was synthesized with
different types of butanediol to modify material properties for bone engineering. Conclusions show that a new acid scavenger free synthesis method creates OPF and PBF with reduced hydrochloric acid and fewer post-processing steps. Oligo(poly(ethylene glycol) fumarate) can be photocrosslinked into hydrogels with positively charged functional groups for encapsulation of valvular cells. Finally, PBF material properties can be chemically controlled by the type of butanediol used and they can be crosslinked into hard plastic films. Overall, the contributions of this dissertation will aid in the development of a new application for currently used biomaterial (OPF) for soft tissue engineering as well as develop and characterize a new variation of PBF for bone tissue engineering.
# Table of Contents

Contents

Abstract................................................................................................................................................... v

Table of Contents ................................................................................................................................... vii

List of Figures ......................................................................................................................................... xii

List of Tables .......................................................................................................................................... xiv

Chapter 1 Review of Fumarate-Based Polymers ................................................................................. 1

1. Origins and Applications of Fumarate .............................................................................................. 1
2. Fumarate-Based Polymers .................................................................................................................. 3
   2.1 Poly(Propylene Fumarate) ............................................................................................................... 3
   2.2 Poly(Propylene Fumarate-co-Ethylene Glycol) ............................................................................. 6
   2.3 Oligo(Poly(Ethylene Glycol) Fumarate) ......................................................................................... 8
   2.4 Poly(Caprolactone Fumarate) ......................................................................................................... 12
   2.5 Poly(Butylene Fumarate) ............................................................................................................... 16
3. Conclusion ......................................................................................................................................... 17

Figures .................................................................................................................................................... 19

References ............................................................................................................................................... 26

Chapter 2 Specific Aims ......................................................................................................................... 39

Objective .................................................................................................................................................. 39

Specific Aims ........................................................................................................................................... 40

Broader Impact ....................................................................................................................................... 41

Chapter 3 Acid Scavenger Free Synthesis of Oligo(Poly(Ethylene Glycol) Fumarate) Utilizing Inert Gas Sparging ......................................................................................................................................... 43
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Authors’ Contribution</td>
<td>44</td>
</tr>
<tr>
<td>Abstract</td>
<td>45</td>
</tr>
<tr>
<td>Impact Statement</td>
<td>46</td>
</tr>
<tr>
<td>Introduction</td>
<td>47</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>48</td>
</tr>
<tr>
<td>Reagents and Chemicals</td>
<td>48</td>
</tr>
<tr>
<td>OPF Synthesis</td>
<td>48</td>
</tr>
<tr>
<td>OPF Characterization</td>
<td>49</td>
</tr>
<tr>
<td>Characterization of Crosslinked OPF Hydrogels</td>
<td>51</td>
</tr>
<tr>
<td>Cytotoxicity Analysis</td>
<td>52</td>
</tr>
<tr>
<td>Statistics</td>
<td>53</td>
</tr>
<tr>
<td>Experiment</td>
<td>53</td>
</tr>
<tr>
<td>Physical Characterization</td>
<td>54</td>
</tr>
<tr>
<td>Increased Oligomerization of OPF Utilizing Nitrogen Sparging Methods</td>
<td>54</td>
</tr>
<tr>
<td>Characteristics of Crosslinked Hydrogels</td>
<td>55</td>
</tr>
<tr>
<td>Cell Viability in the Presents of Polymer Products</td>
<td>56</td>
</tr>
<tr>
<td>Discussion</td>
<td>56</td>
</tr>
<tr>
<td>Conclusions</td>
<td>60</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>61</td>
</tr>
<tr>
<td>Disclosure Statement</td>
<td>61</td>
</tr>
<tr>
<td>Funding Information</td>
<td>61</td>
</tr>
<tr>
<td>Figures and Tables</td>
<td>62</td>
</tr>
<tr>
<td>References</td>
<td>71</td>
</tr>
</tbody>
</table>
Chapter 5 Poly(1,2-Butylene Fumarate) and Poly(1,3-Butylene Fumarate) Characterization for Use in Tissue Engineering

Abstract

Introduction

Materials and Methods

Poly(Butylene Fumarate) Synthesis

Chemical and Material Properties of Poly(Butylene Fumarate)

Poly(Butylene Fumarate) Films

Characterization of Crosslinked Poly(Butylene Fumarate)

Statistics

Results

References
List of Figures

Figure 1-1......................................................................................................................... 19
Figure 1-2.......................................................................................................................... 20
Figure 1-3.......................................................................................................................... 21
Figure 1-4.......................................................................................................................... 22
Figure 1-5.......................................................................................................................... 23
Figure 1-6.......................................................................................................................... 24
Figure 1-7.......................................................................................................................... 25
Figure 3-1.......................................................................................................................... 62
Figure 3-2.......................................................................................................................... 63
Figure 3-3.......................................................................................................................... 64
Figure 3-4.......................................................................................................................... 65
Figure 3-5.......................................................................................................................... 66
Figure 3-6.......................................................................................................................... 67
Figure 3-7.......................................................................................................................... 68
Figure 3-8.......................................................................................................................... 69
Figure 3S-1......................................................................................................................... 79
Figure 3S-2......................................................................................................................... 80
Figure 4-1.......................................................................................................................... 105
Figure 4-2.......................................................................................................................... 106
Figure 4-3.......................................................................................................................... 107
Figure 4-4.......................................................................................................................... 108
Figure 4-5.......................................................................................................................... 109
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td></td>
<td>70</td>
</tr>
<tr>
<td>S3-1</td>
<td></td>
<td>78</td>
</tr>
<tr>
<td>4-1</td>
<td></td>
<td>115</td>
</tr>
<tr>
<td>4-2</td>
<td></td>
<td>116</td>
</tr>
<tr>
<td>5-1</td>
<td></td>
<td>143</td>
</tr>
<tr>
<td>5-2</td>
<td></td>
<td>144</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

1. Origins and Applications of Fumarate

Fumarate is a molecule that is naturally produced and used in the human body. Synthetically derived fumarate-base molecules and polymers have been applied to pharmaceuticals, manufacturing, and, more specifically, tissue engineering applications. Fumarate is a type of butenedioic acid which is a four-carbon molecule containing an $\alpha,\beta$-unsaturated carbonyl group. There are two isomers of butenedioic acid of which fumaric acid is the trans-isomer and maleic acid is the cis-isomer. Fumarate is the salt and ester form of the organic molecule fumaric acid (FA) (Figure 1-1). This molecule contains dicarboxylic acid and $\alpha,\beta$-unsaturated double bonds and is a colorless, odorless, crystalline powder that is found throughout nature but was first isolated and named after the Fumaria officinalis plant. The molecular structure and material properties have made FA useful throughout the food, pharmaceutical, and polymer industry. Federal and Drug Administration (FDA) and World Health Organization (WHO, E297) have found FA practically non-toxic as a food additive for human and animal consumption. It has been used as a cheap acidulant or preservative for beverages and baked goods. It also has a similar sour taste as tartaric acid and citric acid making it an inexpensive flavor enhancer option for foods and drinks.

Found throughout nature, FA is produced in the human body in the citric acid and urea cycles. In the citric acid cycle, succinate is dehydrated through the succinate dehydrogenase enzyme to form FA. In this intermediate step, a flavin adenine dinucleotide
(FADH) is protonated to form an FADH₂, which is a molecule used in cellular respiration. Within hepatocytes, fumarate can be transferred into the cytosol and used in the urea cycle. During the urea cycle, succinate is cleaved from argininosuccinate through the argininosuccinase enzyme. This produces arginine which becomes converted to urea and exported out of the cell, and fumarate which can be recycled to the urea cycle or used in the citric acid cycle.\textsuperscript{5,6} For its vital use in human biology, FA has been investigated as a monomer for creating biomaterials and drugs.

Exploration into the medicinal properties of FA started in the 1950s. An anti-psoriasis medication was first developed using FA esters. Specifically, dimethyl fumarate (DMF) contains two methyl end groups and has been shown to help modulate the autoimmune response during psoriasis flare-ups (Figure 1-1).\textsuperscript{7} Oral delivery of the drug did not readily absorb through the gastrointestinal tract but was hydrolyzed into an ester derivative before entering the circulatory system.\textsuperscript{1,8} Monomethyl fumarate, an ester form of DMF, was able to circulate throughout the body and reduce psoriasis (Figure 1-1). The mode of removal of the fumarate drug was primarily through respiration and a small fraction was through the urinary and fecal routes.\textsuperscript{9} The success of DMF treating psoriasis spurred further investigation of other FA esters for treating the disease. Dimethyl fumarate and diroximel fumarate were found to help mitigate complications due to multiple sclerosis, and formoterol fumarate dihydrate was developed into an inhalant for treating chronic obstructive pulmonary disease and asthma.\textsuperscript{10,11} Fumaric acid esters are continuing to be explored for its medicinal properties.

Fumarate-based molecules have been used in industrial applications as well. Dimethyl fumarate was previously used as a preservative in anti-humidity bags for
transporting furniture and shoes. In the mid-2000s, cases of contact dermatitis were linked to skin exposure to DMF in Europe.\textsuperscript{12,13} Use of DMF as a preservative has recently been limited. Dibutyl fumarate (DBF) is another fumarate-based polymer containing butylene end groups and has been used as a plasticizer (Figure 1-1). Specifically, it can be co-polymerized with other monomers, like vinyl chloride, styrene, acrylate, etc., to prepare coatings, adhesives, and surface conditioning agents.\textsuperscript{14} Additionally, DBF has been investigated as an environmentally friendly plasticizer due to its biodegradable properties in aqueous solutions and low toxicity to mammalian cells.\textsuperscript{15–20}

2. Fumarate-Based Polymers

Fumarate-based polyesters were first developed 20 years ago and used for biomedical applications. They were proposed as an injectable biomaterial that could fill defects and help with tissue repair while avoiding invasive procedures.\textsuperscript{21} Fumaric acid can be synthesized using different monomers to create linear polyesters. With multiple unsaturated doubles bonds in the backbone, fumarate-based polymers are suitable for both thermal and photo crosslinking methods.\textsuperscript{22} Multiple types of crosslinkers can be combined with poly fumarates to control material properties. Depending on the monomer, fumarate can be synthesized to form a hydrophobic polymer like poly(propylene fumarate) (PPF), a hydrophilic polymer like oligo(poly(ethylene glycol) fumarate) (OPF), and amphiphilic polymer like poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)) (Figure 1-2). In this work PPF, P(PF-co-EG), OPF, poly(caprolactone fumarate) (PCLF), and poly(butylene fumarate) (PBF) will be reviewed as they are the most used fumarate-based polymers in tissue engineering.

2.1 Poly(Propylene Fumarate)
Poly(propylene fumarate) (PPF) was first discovered in 1988 and has been shown to have many tissue engineering applications ranging from hard tissue repair (e.g. bone substitute), soft tissue repair, and controlled-release of biological factors, cells, and drugs. Generally, PPF has been shown to have suitable biomaterial characteristics for tissue engineering. Although there have been multiple ways to synthesize PPF, it has often been synthesized in a two-step procedure starting with diethyl fumarate (DEF) and 1,2-propanediol and in the presence of a catalyst. The result of the first step is bis(hydroxypropyl) and ethanol. The second step is a transesterification of bis(hydroxypropyl) followed by a series of aqueous washes to remove the catalyst and remove any unreacted monomers as well as drying and solvent removal steps (Figure 1-3). This method allows for the control of the molecular weight (500-4,000 g/mole) of PPF through the control of heating and reaction time during the transesterification process.

Poly(propylene fumarate) polymer is hydrophobic and highly viscous at room temperature and readily dissolves in chloroform, methylene chloride, tetrahydrofuran, acetone, alcohol, and ethyl acetate. Multiple types of crosslinkers, like poly(ethylene glycol) dimethacrylate (PEGDMA), poly(ethylene glycol) (PEGDA), dimethyl fumarate (DMF), N-vinyl pyrrolidone, and poly(propylene fumarate) diacrylate (PPFDA), have been used to chemically crosslink the polymers into solid materials and control the material properties of the final scaffold. For example, PPF has been combined with PPFDA and showed higher mechanical strength compared to other crosslinkers and increased degradation. This is due to the additional double bond and ester in the PPFDA creating a tighter polymer network and having additional cleavage sites during degradation. Furthermore, PPF and crosslinkers have been crosslinked through thermal and photo
crosslinking methods, making it a versatile material that can be injected into wound sites.\textsuperscript{33–36} Specifically, PPF and DMF have been combined with the photoinitiator bis(2,4,6-trimethylbenzoyl) phenylphosphine oxide (BAPO) and crosslinked with UV light to form casted or printed constructs.\textsuperscript{27} Crosslinked PPF creates a hard plastic material that has similar strength as bone. For instance, crosslinked PPF and DEF materials have an elastic modulus within the 150-200 MPa range.\textsuperscript{27}

Poly(propylene fumarate) has been used as a scaffold material for a variety of cell types but has been primarily explored as a bone scaffold material due to its mechanical properties. Since PPF is made from fumarate and propylene, the degradative byproducts can be excreted from the body through the urinary system, making it safe for implantation into the body (\textit{Figure 1-2}). \textit{In vitro} cell studies have proven that the PPF scaffold supports osteoblastic attachment and bone growth, implying cells can migrate onto the scaffold, attach, and start to replace it with the native extracellular matrix as the scaffold degrades.\textsuperscript{37–39} Stem cells, specifically marrow stromal cells, also successfully grow on PPF scaffolds and can be differentiated into different mesenchymal cells, including osteoblasts, through exogenous factors.\textsuperscript{40,41} \textit{In vivo} studies have shown that PPF bone scaffolds have a mild immunogenic response when implanted, making them an optimal material for medical devices and drug delivery systems.\textsuperscript{24}

Currently, PPF is being used as material for advanced additive manufacturing and drug delivery platforms. Innovations in additive manufacturing are making it possible to create complex three-dimensional (3D) constructs with defined features within the micrometer scale, enabling the replication of native extracellular matrix organization. Bioprinting is a type of additive manufacturing technique that extrudes a material in the form
of a bioink into layers. These layers are then stacked upon themselves to make a 3D construct. Poly(propylene fumarate) has been investigated as a bioink to print porous constructs for bone tissue engineering.\textsuperscript{42–44} Additionally, PPF has been investigated as a material for use as a versatile drug delivery platforms. One application utilizes PPF as a conduit that entraps particles, containing the drug to control the release of the drug and supply a scaffold for tissue regeneration. Specifically, poly(DL-lactic-co-glycolic acid) (PLGA) microspheres were laden with bone morphogenic protein-2 (BMP2) and encased in PPF. The release of BMP2 out of the PLGA microspheres was found to be at a rate proportional to the degradation of PPF and the combination of controlled release of BMP2 and the PPF scaffold were optimal for bone regrowth.\textsuperscript{45–48} The other platform used for drug delivery entails loading PPF scaffold solutions or scaffolds with drugs. This method has been an injectable \textit{in situ} method for repairing tissue and delivering a drug.\textsuperscript{49,50} Additionally, this platform has been employed for 3D bioprinting applications. In one case, a bioink was made with heparin-loaded gelatin methacrylate, decellularized pericardium, and PPF and then printed into layers for vascular engineering materials that are antithrombotic.\textsuperscript{37,51,52} Merging additive manufacturing, drug delivery, and PPF will allow the creation of a bone scaffold that can create the biomechanical, biochemical, and extracellular matrix organization that is necessary to regenerate large defects in bone tissue.

2.2 Poly(Propylene Fumarate-co-Ethylene Glycol)

In an effort to increase the hydrophilicity of PPF, poly(ethylene glycol) has been synthesized with PPF to make P(PF-co-EG) in 1997 to make an amphiphilic polymer (Figure 1-2).\textsuperscript{53–55} Many formations have been derived from the linear block copolymer. Specifically, PEG units of 570, 800, 1,960, and 5,190 g/mole have been combined with PPF
(1,570 g/mole) at a 2:1 PEG:PPF molar ratio. Formulations containing longer chains of PEG show greater hydrophilicity. Traditionally, P(PF-co-EG) has been synthesized using a 3-step procedure. The first two steps are identical to synthesizing PPF while the third step reacted methoxy PEG with PPF under vacuum at 160°C. Purification of the final P(PF-co-EG) polymer was done through filtration and precipitation steps followed by drying under vacuum (Figure 1-4). The final product was either a waxy solid, formulations with less amount of PEG, or a powder, formulations with more amount of PEG. The polymer also contains thermo-sensitive gel properties when dissolved in an aqueous solution and mechanical properties can be improved through crosslinking methods. Specifically, the polymer solution is liquid below temperatures of 25°C and gel above 35°C. The most common method for crosslinking P(PF-co-EG) is through thermal crosslinking with a redox reaction. Ammonium persulfate and N,N,N’,N’-tetramethylethylene diamine, ammonium persulfate and ascorbic acid, or benzoyl peroxide and N-vinyl pyrrolidinone have been used to initiate crosslinking of P(PF-co-EG) into solid structures. It was found that the swelling ratio of crosslinked scaffolds could be reduced by increasing the relative amount of PPF block. Additionally, the mechanical strength of the scaffolds can be decreased by increasing the relative amount of PEG.

Poly(propylene fumarate-co-ethylene glycol) has been developed as a thermoreversible hydrogel platform for delivery cells and bioactive factors to a direct location in the body. This platform has been shown to support chondrocyte viability and growth when encapsulated within. In another study by Suggs and Mikos, P(PF-co-EG) was used as a carrier for endothelial cells injected subcutaneously in a rat’s back. Results demonstrated cells embedded in a thermally crosslinked hydrogel and implanted...
subcutaneously in the rat remained viable. Finally, a normal inflammatory and wound-healing response were observed. Poly(propylene fumarate-co-ethylene glycol) scaffolds have also been investigated for bone engineering by modifying the hydrogel to have cell adhesion ligands. These peptide sequences improved mesenchymal stem cells attachment and spreading while still retaining the ability to differentiate into osteoblasts.

2.3 Oligo(Poly(Ethylene Glycol) Fumarate)

Since its development in 2001, OPF has been extensively used for musculoskeletal, nerve, and other soft tissue applications. The linear polymer consists of multiple poly(ethylene glycol) (PEG) monomers linked together by fumarate groups (Figure 1-2). Traditionally, PEG has been used as a hydrophilic polymer that can be functionalized with acrylates to make hydrogels for a plethora of biomedical applications. Synthesis of OPF was first done using a single-step condensation reaction between PEG and fumaryl chloride in the presence of an acid scavenger, but other methods have been developed to synthesize the polymer without acid scavengers. Briefly, varying molar ratios of PEG to fumaryl chloride (1:0.8, 1:0.9, 1:0.98) were reacted together with an acid scavenger overnight, followed by solvent evaporation and recrystallization steps in ethyl acetate (Figure 1-5A). A newer method for creating OPF has been developed that eliminates the use of any acid scavengers. This method uses nitrogen sparging to mobilize the hydrochloric acid byproducts into a neutralizing bath. This method reduces the necessity for recrystallization and washing post-processing steps (Figure 15B).

The final product at these molar ratios showed that OPF was a solid powder and, depending on synthesis methods, the powder could be light brown, due to residual acid scavenger, or white. Because the reaction is run in an excess of PEG, the OPF molecule
is terminated with PEG end groups which could be utilized for further modifications. Specifically, the hydroxyl end groups of the terminal PEGs can be functionalized with attachment peptides for increased cell attachment.\textsuperscript{66} Furthermore, the molecular weight of the precursor PEG determines much of the physical properties and degree of polymerization of OPF.\textsuperscript{67} Experiments using PEG ranging from 800-30,000 g/mole have been explored for synthesizing OPF. However, molecular weights larger than 10,000 g/mole had less chance of oligomerizing due to steric hindrance of the PEG monomer. It was found that the highest degree of oligomerization of OPF was with 1,000, 3,400, and 4,600 g/mole PEG, which resulted in OPF molecular weights of 7,000, 11,610, 12,600 g/mole, respectfully.\textsuperscript{63}

Since OPF contains multiple unsaturated double bonds and is soluble in water, the polymer can be crosslinked into a network through chemical or photo crosslinking strategies to form a hydrogel system. Thermo crosslinking was traditionally explored as an injectable system that supported a minimally invasive procedure.\textsuperscript{68–71} Ideally, the chemical initiators would crosslink the OPF at body temperature, 37\textdegree\textsuperscript{C}, and within a desired time frame. The gelation time of the OPF solution depended on PEG molecular weight and initiator concentration. Ammonium persulfate and ascorbic acid redox initiators were first used with OPF to form hydrogels. It was found that higher concentrations of initiator were needed to reduce the gelation time from a matter of hours to minutes but the higher concentrations were cytotoxic.\textsuperscript{72,73} Later, PEG diacrylate (PEGDA) was incorporated with the OPF solution and ammonium persulfate/ascorbic acid solution and significantly reduced the crosslinking time.\textsuperscript{74} Due to the cytotoxicity of ammonium persulfate and ascorbic acid, other redox initiators have been investigated — namely, ammonium persulfate and N,N,N’,N’-tetramethylethylendiamine (TEMED) or sodium persulfate with sodium ascorbate and
magnesium ascorbate-2-phosphate.\textsuperscript{72,73} Photo crosslinking has also been a successful method for crosslinking OPF for cell growth, cell encapsulation, and drug delivery applications. Multiple types of initiators, including Irgacure 819, Irgacure 2959, and Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), have been used to make hydrogels.\textsuperscript{75–79} Concentrations ranging from 0.05\%-0.3\% (w/w) of Irgacure 2959 were found to be crosslinked within a time frame of 10-40 minutes under UV exposure (365 nm). The more water soluble photoinitiator LAP was found to reduce the UV exposure time for crosslinking OPF and PEGDA hydrogels and was less cytotoxic.\textsuperscript{77,78} Finally, the addition of crosslinkers, like PEGDA, accelerated the photocrosslinking.

Mechanical properties of crosslinked OPF hydrogels were highly dependent on the molecular weight of the PEG monomer and the degree of oligomerization of OPF. Specifically, lower molecular weight PEG and higher oligomerized OPF increased tensile and compression strength.\textsuperscript{70,71,74} This phenomenon is attributed to the mesh size of the polymer network increasing when PEG molecular weight increases and few fumarate groups are available for crosslinking.\textsuperscript{63,74,80,81} Additionally, degradations studies of crosslinked OPF hydrogels showed that crosslinking density influenced \textit{in vivo} biocompatibility. In subcutaneous rabbit studies, OPF8K degraded faster causing a higher number of inflammatory cells to surround the fragmented OPF hydrogel, while OPF1K degraded slower reducing the inflammatory response.\textsuperscript{71}

Oligo(poly(ethylene glycol) fumarate was first developed for cartilage and bone regeneration and was shown to successfully support \textit{in vitro} cell growth of native cells on and within hydrogels as well as differentiation of bone marrow-derived stem cells into chondrocytes and osteoblasts. Through the addition of attachment peptides, the hydrogel
platform also showed minimal inflammatory activation while promoting cell migration and growth in the scaffolding during *in vivo* studies.\(^{71,80,82}\) More advanced OPF platforms have started incorporating timed-release growth factors or bioactive particles components to create an environment that promotes either cartilage or bone tissue. Such growth factors like transforming growth factor-β1 (TGF-β1) have been shown to promote cartilage tissue growth through the production of glycosaminoglycans (GAG), collagen II, and aggrecan.\(^{70,83–85}\) Bioactive particles have also been incorporated into the hydrogel matrix to improve bone formation. Calcium phosphate was first incorporated in OPF hydrogels to help stimulate bone remodeling *in vivo* studies.\(^{86–89}\)

Furthermore, OPF has been investigated as a biomaterial for nerve conduits to regenerate nerves. Specifically, electrically charged OPF hydrogels have been shown to improve nerve repair.\(^{90,91}\) Although very little research has been performed on anionic OPF hydrogels, there have been several studies using cationic moieties in OPF hydrogels. Dadsetan *et al*. created a positively charged hydrogel platform with OPF and [2-(methacyloyloxy)ethyl]-trimethylammonium chloride (MAETAC) to demonstrate that positively charged OPF (OPF+) hydrogels had a dose-dependent effect on neurite outgrowth.\(^{92–94}\) Specifically, OPF+ containing 400 mM and 600 mM of MAETAC supported more outgrowth of dorsal root ganglia, attachment of Schwann cells, and neurite myelination compared to concentrations lower than 400 mM and 800 mM. Moreover, multi-channel cylindrical OPF+ conduits implanted in the transected spinal cord of rats resulted in better tissue interaction and integration to the point of filling the transected gap and structural and functional restoration. It was also found that these conduits reduced the amount of spinal cyst and scar tissue formation.\(^{95,96}\) Other tissue engineering and regenerative applications have
been explored using OPF. Oligo(poly(ethylene glycol) fumarate) has been explored as a biomaterial for pigment epithelial cell delivery for lens regeneration as well as ligament scaffolding for ligament regeneration.\textsuperscript{97–100} Finally, conductive OPF polymers have been investigated as a biomaterial that can reestablish the electrical connection of damaged cardiomyocytes and promote neovascularization.\textsuperscript{101,102}

2.4 Poly(Caprolactone Fumarate)

Poly(caprolactone) (PCL) is an FDA approved hydrophobic polymer that is widely used as a biomaterial for sutures and drug delivery applications.\textsuperscript{103,104} This synthetic polymer is one of the most studied and used biomaterials for biomedical applications. Poly(caprolactone fumarate)’s molecular weight and degradation rate can be used to control the polymer’s physicochemical properties.\textsuperscript{105} Specifically, PCL usually degrades over 2-4 years which is a significant time frame for tissue engineering applications.\textsuperscript{103,106,107} Therefore, copolymerization with fumarates has been developed to create PCLF, a faster degrading aliphatic polymer.\textsuperscript{108,109} The addition of the unsaturated double bonds and the flexibility of the polymer’s backbone due to the long caprolactone chains allows PCLF to self-crosslink without the addition of crosslinkers, ideal for \textit{in situ} crosslinking which is attractive for tissue engineering and injectable delivery systems.\textsuperscript{108,109} Poly(caprolactone fumarate) was first reported synthesized in 2005 through a one-step polycondensation polymerization of PCL diols with fumaryl chloride in the presence of an acid scavenger. Briefly, PCL diols were dissolved in dichloromethane and fumaryl chloride, and an acid scavenger was added dropwise to the reaction. The final product was then post-processed through precipitation in petroleum ether and dried under vacuum (\textbf{Figure 1-6}). Varying molecular weights of PCL (530, 1,250, 2,000 g/mole) were shown to form PCLF and the
final product was in the form of wax- or past-like texture. This method by Jabbari et al. has been widely adopted.\textsuperscript{108–110} Further adoptions to the type of acid scavenger were shown to improve the coloration of the final polymer as well as a two-step synthesis process utilizing a ring-opening step of caprolactone followed by the condensation polymerization of PCL with fumaryl chloride, which was shown to reduce the cytotoxic byproduct of diethylene glycol.\textsuperscript{110–112}

It was found that the molecular weight of the precursor PCL affected the physical properties of the PCLF. As the molecular weight of PCL increased, the crystallinity and melting point also increased.\textsuperscript{39,109,113} A similar trend was found for the thermal stability of PCLF. As PCL molecular weight increased the degradation onset temperature increased making PCLF with higher molecular weight PCL more thermally stable.\textsuperscript{114} Furthermore, Wang et al. showed the PCLF did not start hydrolytically degrading until week 20 at 37℃ in PBS, despite the molecular weight of PCL monomer. It was not until week 71 that the higher molecular weight PCLF was mostly degraded.\textsuperscript{114} As a biomaterial for tissue engineering, the incorporation of fumarate in PCLF reduced the degradation time of traditional PCL.

Since PCLF can crosslink, thermal and photo crosslinking strategies have been investigated as methods to create scaffolds. Like other thermal crosslinking methods, a crosslinker and chemical initiator was combined with PCLF. Crosslinkers investigated have been methyl methacrylate, PEGDA, and N-vinyl pyrrolidone with chemical initiators benzoyl peroxide and N-dimethyl toluidine.\textsuperscript{109} Temperatures within the body temperature (37-40℃) were able to crosslink PCLF solutions.\textsuperscript{108,115} Additionally, composites PCLF scaffolds using hydroxyapatite particles resulted in crosslinking with higher temperatures of 90℃ and an additional post-curing step at 120℃.\textsuperscript{116} The more common method of crosslinking PCLF has
been photocrosslinking because it is simple, requires less time, and circumvents the use of several toxic monomers and initiators needed for thermocrosslinking.\textsuperscript{67} Similar to PPF photocrosslinking strategies, PCLF has been combined with multiple photoinitiators. The primary initiator used has been bisacylphosphinoxide (BAPO). Varying concentrations of BAPO were tested to determine the degree of crosslinking. These tests showed that the gel fraction increased and the swelling ratio decreased with the increase in BAPO concentration. Additionally, the PCL molecular weight affected the swelling ratio with the highest molecular weight PCL causing the highest swelling ratio of PCLF.\textsuperscript{109} In an attempt to use a more biocompatible photoinitiator system, Sharifi \textit{et al.} used camphorquinone and N, N’-diemthyl aminoethylemethacrylate and concluded that PCLF scaffolds could be formed with blue light, rather than UV light, and shorter crosslinking times (~4 minutes), compared to an hour of UV exposure.\textsuperscript{117,118}

Poly(caprolactone fumarate) scaffolds have been primarily used in nerve and bone tissue engineering applications, with some studies applying PCLF to ligament, cartilage, and blood vessel engineering. Nerve conduits have been developed using PCLF and have shown success in growing and repairing nerves. Initially, PCLF conduits attached to nerves and supported a high number of myelinated axons through the cylinder with minimal fibrosis after 6 and 17 weeks.\textsuperscript{114} Then, multi-channeled nerve conduits that delivered Schwann cells were used as a conduit for a transected rat spinal cord. Results show that significant growth of axons in random directions was observed within the channels. The high compressive moduli of the PCLF did promote the formation of cyst and scar tissue in the rostral and caudal interface of the conduit with the spinal cord.\textsuperscript{95} Furthermore, PCLF has been investigated as a scaffolding material for growing fully functional nerve grafts. In order to
make nerve grafts researchers have created charged PCLF scaffolds using polypyrrole with five different anions and conductive carbon nanotubes.\textsuperscript{94,119–121} It was found that the increase in conductive capacity improved the proliferation, neurite outgrowth, neuronal differentiation, and the formation of synapse-like intercellular connections of PC-12.\textsuperscript{120–122}

As a scaffold for bone regeneration, PCLF has a lower modulus for loading bearing bone. Therefore, a lot of research has aimed at creating composite materials using PCLF and bioceramics. It was shown that incorporating hydroxyapatite nanoparticles increased the compressive modulus of PCLF with lower molecular weight PCL on an order of 3.5x with the addition of 30\% of hydroxyapatite nanoparticles but did not improve the compressive strength of PCLF with higher molecular weight PCL. The hydroxyapatite nanoparticles improved bone marrow stem cell attachment and proliferation.\textsuperscript{116,123} Moreover, PCLF was used as a coating solution into porous hydroxyapatite scaffolds. This method increased the overall mechanical strength of the scaffold but PCLF had little effect on osteoblast attachments as they already liked to attach to the hydroxyapatite scaffold and possibly inhibited cell attachment and proliferation because of PCLF hydrophobic properties.\textsuperscript{124–127}

Woven PCLF fibers with poly(ethylene terephthalate) (PET) were investigated as a ligamentous scaffold for anterior cruciate ligament (ACL) repair. Mechanical testing of hybrid scaffolds resulted in a failure load of 72 N which is far less than the native ACL failure load of 353 N.\textsuperscript{128} \textit{In vivo} studies showed that after 8 weeks the mean failure loads decreased for PCLF-PET scaffolds and was disintegrated into pieces. These pieces resulted in a dense inflammatory reaction making PCLF not an ideal candidate for ligament engineering.\textsuperscript{128} Additionally, PCLF has been investigated as cartilage and soft tissue scaffolding. It was shown that PCLF supports cell growth and production of key
cartilaginous markers but due to the hydrophobic nature of the polymer, growth factors could not be loaded into the scaffold to improve tissue formation.\textsuperscript{129} The combination of PCLF and growth factor-loaded PLGA microspheres delivered the growth factors in a controlled manner and resulted in infiltration of tissues and neovascularization into the scaffold.\textsuperscript{130}

2.5 Poly(Butylene Fumarate)

Poly(butylene fumarate) is a linear polyester that has similar properties to PPF. Like PPF, PBF is a hydrophobic polymer that contains unsaturated double bonds and is hydrolytically biodegraded, but unlike PPF, it can be chemically engineered to have different material properties through manipulation of its butylene group. In early 2010, two forms of PBF were first reported and they were synthesized with 1,3-butanediol or 1,4-butanediol (Figure 1-2).\textsuperscript{131−133} The linear form of PBF was developed into a block copolymer with poly(butylene succinate) and poly(butylene maleate).\textsuperscript{133−136} Multiple synthesis methods have been used to make PBF. One method used a ring-opening strategy starting with maleic anhydride where maleic anhydride, 1,3-butanediol, and an acid scavenger were heated together to 250°C for 3 hours. This synthesis process starts by converting maleic anhydride to maleic acid. Followed by the transformation of maleic acid into fumaric acid. The transesterification of fumaric acid and 1,3-butanediol then proceeds. The final crude polymer was post-processed by washing and drying steps (Figure 1-7).\textsuperscript{132,133,136} The other synthesis methods used a two-step, esterification, and polycondensation, process. Briefly, fumaric acid, 1,4-butanediol, an acid scavenger, and a free radical inhibitor were reacted together at 150°C until all the water byproduct was removed. The first step produced PBF-diol which is then put under pressure (5-15 Pa) and polymerized to create specific molecular weights of PBF.
**Figure 1-7** PBF is a viscous brownish material that is highly tactile when synthesized with 1,3-butanediol or a solid material if synthesized with 1,4-butanediol.

Since the PBF contains unsaturated double bonds, it has been developed into a crosslinkable material. Specifically, the poly(1,3-butylene fumarate) (1,3-PBF) has been developed into a photocrosslinkable material using BAPO and UV light. The crosslinked 1,3-PBF scaffolds have shown similar mechanical properties as PPF and were appropriate for orthopedic applications. It was concluded that the addition of the methylene unit (-CH₂) also increased the degradation of 1,3-PBF relative to PPF. The poly(1,4-butylene fumarate) (1,4-PBF) was found to be a crystalline polymer forming solids at room temperature. It was found that the tensile and flexural strength of 1,4-PBF were 41.0 MPa and 26.7 MPa.

Poly(butylene fumarate) has not been thoroughly investigated as a crosslinkable biomaterial for biomedical applications, which offers an opportunity for further development as a tissue engineering polymer. Current uses of PBF for biomedical applications have investigated poly(1,3-butylene fumarate) (1,3-PBF) as a biodegradable polymer for bone engineering. It was found that 1,3-PBF scaffolds were not cytotoxic to bone marrow derived mesenchymal stem cells and supported cell attachment and differentiation into osteoblasts. Additionally, 1,3-PBF has been used to create electrospun mats that have potential as a scaffolding material and drug delivery system.

### 3. Conclusion

Fumarates are a versatile biomolecule that the human body has utilized for creating energy and expelling waste. Scientists and engineers have discovered the medicinal and industrial properties of fumarates but more recently have created polymers useful for
biomedical applications. Recent developments in fumarate-based polymers have created a class of polymers, PPF, P(PF-co-EG), OPF, PCLF, and PBF, with ranging material properties. Their past and present applications in musculoskeletal, nerve, and other tissue applications have shown these biomaterials as useful biodegradable scaffolds capable of drug delivery. As tissue engineering progresses, fumarate-based polymers can be used for solving future challenges of growing complex tissues, modeling cell environments, and developing new drugs and treatments that will be used to progress the regenerative medicine field.
Molecular structures of fumarate-based small molecules. Fumaric acid is a vitally important molecular found in the human body. Fumaric acid esters, dimethyl fumarate, diethyl fumarate, diroximel fumarate, are pharmaceuticals that are cleaved within the body to create the pharmacologically active monomethyl fumarate. Dibutyl fumarate is an industrial plasticizer.

Figure 1-1. Molecular structures of fumarate-based small molecules. Fumaric acid is a vitally important molecular found in the human body. Fumaric acid esters, dimethyl fumarate, diethyl fumarate, diroximel fumarate, are pharmaceuticals that are cleaved within the body to create the pharmacologically active monomethyl fumarate. Dibutyl fumarate is an industrial plasticizer.
Figure 1-2. Molecular structure of fumarate-based polymers used in tissue engineering, regenerative medicine, and drug delivery.
Figure 1-3. Synthesis of PPF in a two-step polycondensation method. Step one reacts DMF and propylene glycol to make short oligomers of bis(hydroxypropyl) fumarate. The second step is a transesterification process that creates long chains of PPF. Adapted from (28)
Figure 1-4. Synthesis of P(PF-co-EG) in a three-step process. The first two steps are identical to synthesizing PPF. The third step is a transesterification of PEG with PPF. Adapted from (56)
Figure 1-5. Two methods for synthesizing OPF. (A) is a single-step condensation reaction that uses an acid scavenger to capture hydrochloric acid byproducts. (B) is another single-step condensation reaction but uses inert gas sparging to mobilize hydrochloric acid byproduct into a base bath. Adapted from (65)
Figure 1-6. One-step polycondensation reaction for synthesizing PCLF. Varying molecular weights of PCL reacted with fumaryl chloride to create varying molecular weights of PCLF. Adapted from (108)
**Ring-Opening Synthesis**

\[
\text{Maleic Anhydride} + 1,3\text{-Butanediol} \xrightarrow{\text{Catalyst} 250^\circ C} \text{Poly(1,3-Butylene Fumarate)}
\]

**Two-Step Synthesis**

(a)

\[
\text{HO} = \text{C} = \text{O} + \text{HO} - \text{C} - \text{C} - \text{OH} \rightarrow -\text{H}_2\text{O} \rightarrow \text{HO} - \text{C} - \text{C} - \text{O} - \text{C} - \text{C} - \text{O} - \text{C} - \text{C} - \text{O} - \text{H}
\]

PBF-Diol

(b)

PBF-Diol $\xrightarrow{\text{HDI}}$ HDI $\xrightarrow{\text{Chain-Extension}}$

Figure 1-7. Two synthesis strategies used to synthesize PBF. Ring-Opening synthesis has been used to create 1,3-PBF. The two-step synthesis uses an esterification step (a) to create 1,4-PBF (PBF-Diol). Followed by a polycondensation step (b) to create long chains of 1,4-PBF. Adapted from (133) and (131)
References


(14) Llanes, L. C.; Clasen, S. H.; Pires, A. T. N.; Gross, I. P. Mechanical and Thermal Properties of Poly(Lactic Acid) Plasticized with Dibutyl Maleate and Fumarate Isomers:


(91) Fine, E. G.; Valentini, R. F.; Bellamkonda, R.; Aebischer, P. Improved Nerve Regeneration through Piezoelectric Vinylidenefluoride-Trifluoroethylene Copolymer


Chapter 2

Specific Aims

Objective

The tissue engineering paradigm uses three factors, biomaterials, growth and soluble factors, and cells, to design and replicate complex tissue systems for pharmaceuticals, medical devices, and regenerative medicine development. Natural and synthetic biomaterials are typically used as scaffolding for cells to attach, proliferate, and grow into specific types of tissue. The chemical and mechanical properties of the scaffolds are a factor that can be used to direct cells into specific tissue types. For instance, soft tissue highly prefers soft scaffolds, while hard tissue prefers porous, stiff scaffolds.

Traditional culturing methods on tissue culture polystyrene surfaces have been shown to alter or direct the aortic valvular interstitial cell (VIC) phenotype. Specifically, it was found that positively charged self-assembled monolayers can direct VICs to become osteoblastic-like, the major phenotype that leads to aortic stenosis. The fumarate-based polymer oligo(poly(ethylene glycol) fumarate) (OPF) is a hydrophilic polymer with multiple unsaturated bonds useful for crosslinking or functionalizing. This makes OPF advantageous for making a cell platform that mimics the soft tissue of the aortic heart valve leaflets and exposes VICs to a three-dimensional (3D) environment. Therefore, a positively charged OPF hydrogel has been investigated to determine if positively charged environments stimulate VICs to become osteoblastic-like.

Current research is investigating how to make composite scaffolds that replicate the biomechanical, biochemical, and material organizations of the bone-ligament (BL) interface. Specifically, the BL milieu transitions from a hard crystalline bone phase through a
fibrocartilaginous enthesis into an elastic fibrous ligament phase. Poly(propylene fumarate) (PPF), another fumarate-based polymer, has been used extensively as a biomaterial for growing bone. Similar to PPF, poly(butylene fumarate) (PBF) is a hydrophobic polymer that also contains multiple unsaturated bonds but, unlike PPF, PBF has the ability to be chemically engineered to have varying material properties by using different conformations of butylene. The different forms of PBF can be photocrosslinked into constructs with varying mechanical and chemical properties which potentially can be useful for creating the BL interface. Therefore, poly(1,2-butylene fumarate) (1,2-PBF) and poly(1,3-butylene fumarate) (1,3-PBF) has been investigated as polymers used for creating the BL interface.

In this work, fumarate-based biomaterials have been investigated as soft and hard biomaterials for tissue engineering. We showed that both OPF and PBF (1,2-PBF and 1,3-PBF) can be synthesized using an acid-free synthesis method developed in our lab. Additionally, OPF hydrogels have been investigated as a 3D culturing platform for testing positively charged 3D environments on VICs. Finally, two variations of PBF, 1,2- and 1,3-PBF, have been synthesized and characterized.

**Specific Aims**

The following specific aims throughout this project:

*Specific Aim 1*: Fabricate a synthetic OPF-based hydrogel with tunable positively charged densities.

It is hypothesized that a polymer concentration of 25 wt% of OPF and crosslinker will form hydrogels with mechanical properties similar to soft tissue and have the ability to support varying concentrations of charged monomers up to a concentration of 400 mM.
Specific Aim 2: Characterize the viability of the valvular interstitial cells (VICs) when exposed to the positively charged OPF hydrogel solution, during UV encapsulation, and in culture through fluorescent assay analysis.

It is hypothesized that VICs will be encapsulated in the positively charged environment and the hydrogels with higher charge density will cause VICs to present a diseased osteoblastic-like phenotype.

Specific Aim 3: Investigate the chemical and material properties of 1,2-PBF and 1,3-PBF polymers and crosslinked films.

It is hypothesized that 1,3-PBF will have properties similar to bone tissue and 1,2-PBF will have properties useful for growing mineralized fibrocartilage tissue.

Broader Impact

Upon completion of this work, two fumarate-based polymers will have been applied to two different tissue engineering applications. First, using OPF to make a hydrogel system for encapsulating and growing VICs will create a 3D cell culture platform for valvular engineering. Incorporating positively charged end groups into the hydrogel network proves the feasibility of using charged environments for further cell-material interactions studies. This will help to develop smarter materials for medical applications. Second, PBF will be investigated as a stiff biomaterial. Two variations of PBF will have been synthesized and characterized. Specifically, 1,2-PBF has never been synthesized and investigated as a biomaterial for tissue engineering and will be considered as a new biomaterial for engineering the biomechanical BL interface. Potentially, 1,2-PBF and 1,3-PBF will have application in developing a BLB construct for ligament repairs. Further research and application of fumarate-based polymers show their utility not only in the tissue engineering
and regenerative medicine field but also as biomaterials for additive manufacturing applications, cell and organ modeling, and medical device development.
Chapter 3

Acid Scavenger Free Synthesis of Oligo(Poly(Ethylene Glycol)Fumarate)

Utilizing Inert Gas Sparging

Matthew N. Rush, PhD,1–3 Kent E. Coombs, PhD,1,4 Christian T. Denny, MS,1,5 David Santistevan, BS,1 Quan M. Huynh, MS,1,5 Kirsten N. Cicotte, PhD,1,5 and Elizabeth L. Hedberg-Dirk, PhD1,2,5,6

1Center for Biomedical Engineering, University of New Mexico, Albuquerque, New Mexico, USA.
2Nanoscience and Microsystems Engineering Graduate Program, University of New Mexico, Albuquerque, New Mexico, USA.
3Center for Integrated Nanotechnologies, Sandia National Laboratories/Los Alamos National Laboratory, Albuquerque, New Mexico, USA.
4Biomedical Sciences Graduate Program, University of New Mexico, Albuquerque, New Mexico, USA.
5Biomedical Engineering Graduate Program, University of New Mexico, Albuquerque, New Mexico, USA.
6Department of Chemical and Biological Engineering, University of New Mexico, Albuquerque, New Mexico, USA.

Published: TISSUE ENGINEERING: Part C
DOI: 10.1089/ten.tec.2021.0027
Correspondence Author: Elizabeth L. Hedberg-Dirk
Address: Center for Biomedical Engineering
MSC01 1120,
1 University of New Mexico
Albuquerque, NM 87131, USA
Email: edirk@unm.edu

Alternate Author Email:
Matthew N. Rush, Matthew.Rush@nau.edu
Kent E. Coombs, coombske@lanl.gov
Christian T. Denny, denny@unm.edu
David Santistevan, dasantistevan@salud.unm.edu
Quan M. Huynh, quanh@unm.edu
Kirsten N. Cicotte, kirsten.cicotte@gmail.com

* Parts of this manuscript first appeared in the PhD thesis of Kent E. Coombs, “Designing synthetic environments to control valvular interstitial cells in vitro,” May 1, 2018 (https://digitalrepository.unm.edu/biom_etds/178).
Authors’ Contributions

M.N.R. wrote and edited the article. K.E.C., C.T.D., D.S., Q.M.H., K.N.C., and E.L.H-D. reviewed the article and provided feedback. All authors agreed on the final version of the article.

Author C.T.D. contributed significant work in characterizing the mechanical properties of materials through compression testing, data analysis, and written sections (Materials and Methods, Results, Discussion). Details are provided in a supplemental section at the end of this chapter.
Abstract

The macromolecule oligo(poly(ethylene glycol) fumarate) (OPF) exhibits promising attributes for creating suitable three-dimensional hydrogel environments to study cell behavior, deliver therapeutics, and serve as a degradable, nonfouling material. However, traditional synthesis techniques are time consuming, contain salt contaminants, and generate significant waste. These issues have been overcome with an alternative, one-pot approach that utilizes inert gas sparging. Departing from previous synthetic schemes that require acid scavengers, inert gas sparging removes byproducts in situ, eliminating significant filtration and postprocessing steps, while allowing a more uniform product. Characterized by nuclear magnetic resonance, gel permeation chromatography, and differential scanning calorimetry, nitrogen sparge synthesis yields an OPF product with greater polymer length than traditional acid scavenger synthesis methods. Furthermore, nitrogen-sparged OPF readily crosslinks using either ultraviolet or thermal initiator methods with or without the addition of short-chain diacrylate units, allowing for greater tunability in hydrogel properties with little to no cytotoxicity. Overall, inert gas sparging provides a longer chain and cleaner polymer product for hydrogel material studies while maintaining degradable characteristics.

Keywords: oligo(poly(ethylene glycol) fumarate), OPF, polyethylene glycol, polymers, hydrogel
**Impact Statement**

Using nitrogen sparging, we have demonstrated that oligo(poly(ethylene glycol) fumarate) (OPF) can be produced with decreased postprocessing, increased product purity, greater oligomerization, and cell viability. These properties lead to greater tunability in mechanical properties and a more versatile hydrogel for biomedical applications. The simplification of synthesis and elimination of impurities will expand the utility of OPF as a degradable hydrogel for cell culture, tissue engineering, regenerative medicine, and therapeutic delivery, among other applications.
Introduction

Oligo(poly(ethylene glycol) fumarate) (OPF) has been extensively investigated as a biodegradable hydrogel for use in tissue-engineered scaffolds and drug delivery.\textsuperscript{1–13} The step-growth polymerization of polyethylene glycol (PEG) and fumaryl chloride (FuCl) produces a linear polyester with hydrochloric acid (HCl) byproducts. The resulting oligomer consists of a repeating scheme of PEG and fumarate, which can be chemically crosslinked into a nonfouling hydrogel (Figure 3-1). The ability of OPF to undergo hydrolytic degradation through ester hydrolysis gives OPF greater utility over previous polyethylene glycol diacrylate (PEGDA) hydrogels.\textsuperscript{14–17} In addition, the physical properties of OPF can be easily tailored by changing the PEG length.\textsuperscript{1,18} Due to the presence of unsaturated double bonds, functionalization of the oligomer backbone can be achieved before or during crosslinking with the incorporation of short-chain molecules.\textsuperscript{4,10} Overall, these factors make OPF a desirable synthetic polymer for multiple applications.

Developed by Jo et al., OPF synthesis has not been significantly modified since its inception.\textsuperscript{1,2,19} However, several groups have reported issues with removal of HCl byproducts affecting crosslinking efficiency and cell viability.\textsuperscript{20–22} To trap HCl, groups have used acid scavengers such as triethylamine (TEA), potassium carbonate, and sodium hydroxide.\textsuperscript{16,23–28} However, batch-to-batch inconsistencies in polymer product are common due to inefficient removal of insoluble HCl-acid scavenger salts.\textsuperscript{22}

Common in waste treatment and manufacturing procedures, sparging is a fast and efficient method easily introduced into the processing environment.\textsuperscript{29–33} By bubbling a chemically inert gas such as nitrogen through a liquid, sparging removes low boiling-point solutes in real-time.\textsuperscript{32,33} Furthermore, the introduction of compressed gas into the system
serves as a heat sync through adiabatic expansion, maintaining the low temperatures required for efficient step-growth polycondensation.\textsuperscript{34} Through the introduction of gas sparging, we have developed a one-pot, straightforward, and improved method for the production of OPF while simultaneously eliminating the complications of acid removal, filtration, and postprocessing.

**Materials and Methods**

*Reagents and chemicals*

PEG (M\textsubscript{n} 4600), FuCl, ascorbic acid (AA), Irgacure 2959, trimethylamine, PEGDA (M\textsubscript{n} 575), deuterated chloroform, and tetrahydrofuran were purchased from Sigma-Aldrich. Ammonium persulfate (APS; ACS grade) and ethanol along with cell culture reagents, Dulbecco’s minimum essential growth medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin–streptomycin, fungizone, trypsin–ethylenediaminetetraacetic acid (EDTA), and live/dead cell viability kit were purchased from ThermoFisher Scientific. Dichloromethane (DCM), ethyl ether, ethyl acetate, anhydrous toluene (99.8\%), and sodium hydroxide were purchased from EMD Millipore.

*OPF synthesis*

PEG drying. PEG was dried by azeotropic distillation before use as previously described.\textsuperscript{2} In brief, 100 g of PEG was added to 200 mL of toluene. At least 150 mL of toluene and residual water were distilled off at 200°C. Toluene was removed on a rotary evaporator followed by drying *in vacuo* overnight. Dried PEG was stored in a desiccator until used.

OPF oligomer synthesis—TEA-OPF. TEA-OPF was prepared according to previously described methods.\textsuperscript{1,2} In brief, dried PEG (100 g) was dissolved in 700 mL of DCM
in a 1 L three-neck round bottom flask set in an ice bath (Figure 3-1A). Air was removed and replaced with a nitrogen environment. Using molar ratios of 1:0.9 PEG to FuCl and 1:2 FuCl to TEA, FuCl and TEA were dissolved in 60 mL DCM in separate addition funnels and simultaneously added dropwise to PEG solution over 4 h. The reaction was stirred vigorously. Upon complete addition of FuCl and TEA, the reaction mixture was continued over an ice bath overnight, followed by solvent removal in vacuo. The product was subsequently dissolved in warm ethyl acetate, chilled (-2°C) to recrystallize OPF and filtered to remove TEA-HCl salts (3x). After the final collection, the OPF was washed with ethyl ether and fully dried under reduced pressure overnight.

**OPF oligomer synthesis—nitrogen sparging.** Dried PEG (100 g) was dissolved in 700 mL DCM in a three-neck round bottom flask with a stir bar and nitrogen environment. Sparging with nitrogen was introduced through central arm with a 24/40 adaptor fitted with a fine-fritted gas dispersion tip and forced out through 90° flow control adapter into a secondary vessel containing 10 wt% sodium hydroxide in ethanol (Figure 3-1B). FuCl was dissolved in 60 mL DCM at a molar ratio of 1:0.9 PEG to FuCl and added dropwise to the PEG solution over 4 h with vigorous stirring. Upon complete addition of FuCl to PEG solution, DCM was added to the reaction mixture to bring the volume back up to 700 mL. The reaction was allowed to progress overnight with sparging followed by solvent removal in vacuo.

**OPF characterization**

OPF structure. OPF products were characterized by hydrogen nuclear magnetic resonance (¹H NMR) 300M Hz (Bruker Avance III 300; TopSpin V3.5) in deuterated chloroform. Peaks were assigned to confirm the hydrogen binding environment. The degree
of polymerization was assessed through peak integration comparing olefin (6.87 ppm) and PEG (3.38–3.85 ppm) functional groups.

Molecular weight and oligomerization. Molecular weight of OPF was determined through gel permeation chromatography (GPC). Styrage HR 4 + 4E, 7.8 · 300 mm (WAT044225 and WAT044240) columns were used to elute the samples at a 1 mL/min flow rate on an Agilent 1100 Series high-performance liquid chromatography system (Hewlett Packard, RID G1362A; Chemstation B.04.01). Four to five samples of 3 mg were run in 1 mL of tetrahydrofuran. Sample weight-average (M$_{w}$) and number-average molecular weights (M$_{n}$) were calculated compared to PEG standards (PL2070-194, 440, 600, 1080, 1470, 4100, 7100, 12,600, 23,600; Polymer Laboratories/Agilent Technologies). The degree of oligomerization ($X_n$) was determined using Equation (1):

$$X_n = \frac{M_{n,OPF}}{M_{n,PEG}},$$

here, M$_{n}$, OPF and M$_{n}$, PEG represent the number-average molecular weight (M$_{n}$) of OPF, and monomeric PEG as determined by GPC.

Changes in molecular weight over time were measured by removing a small volume (1 mL) of OPF product from both TEA-OPF and nitrogen-sparged OPF (N$_2$-OPF) synthesis reaction vessels over the initial 14 h of synthesis in parallel reactions. Solvent was removed in vacuo and 3 mg of product were suspended in 1 mL tetrahydrofuran. Refractive index intensity was normalized to the highest peak of the eluted product.

Fluorescent byproduct. Fluorescence of the polymer product was detected during GPC through the use of a fluorescence detector (Hewlett Packard, FLD G1321A, Ex 250 nm, Em 410 nm) upstream of the refractive index detector; retention time difference 1 min.
Melting temperature and crystallinity. Differential scanning calorimetry (DSC; TA instruments model 2920, Netzsch Proteus) was carried out to determine the crystallinity and melting properties of OPF. Samples were analyzed at a heating rate of 10°C/min from 0°C to 70°C. Melting temperature and the heat of fusion, $D H_m$ (cal/g), were obtained from the thermograms. The percent crystallinity ($X$) of OPF was determined from the following equation:

$$X = \frac{D H_m}{D H_m^*} \times 100,$$

here, $H_m^*$, is the theoretical heat of fusion of 100% crystalline PEG (49 cal/g). \(^{35}\)

Percent yield. Percent yield was calculated by weighing the product recovered from the reaction and dividing it by the weight of reagents originally added.

$$\% \text{ Yield} = \frac{\text{weight OPF final product}}{\text{weight PEG} + \text{weight Fumaryl chloride}}; \quad (3)$$

Characterization of crosslinked OPF hydrogels

Crosslinking of OPF. OPF hydrogel products were crosslinked with and without PEGDA (Sigma Aldrich). The ultraviolet (UV) crosslinked formulations were as follows: 25% (wt%) OPF, 0.5% Irgacure 2959 initiator, 74.5% deionized water or 16.5% OPF, 8.5% PEGDA, 0.5% Irgacure 2959 initiator, and 74.5% deionized water. UV formulations were exposed to 365 nm UV light in a CL-1000 lightbox (UltraViolet Products, UVP) for 15 min. The thermally crosslinked hydrogels had the same formulations stated above, except that 17.6 $\cdot$ 10^{-6} wt% (0.1 mM) AA with 22.8 $\cdot$ 10^{-6} wt% (0.1 mM) APS was added in place of Irgacure 2959. Polymer solutions (1 mL) were transferred to syringe molds before crosslinking. \(^{21}\) Thermally initiated formulations were placed in the incubator (37°C) for 15
min to form hydrogels. Final products were dried overnight at room temperature before being placed in the vacuum oven for 24 h.

Swell ratio and sol fraction. Hydrogels were weighed after drying in a vacuum oven to measure the initial mass of polymer ($W_i$). Hydrogels were then soaked in 5 mL of deionized water and allowed to swell. After 4 days, the maximum swollen weight ($W_s$) was recorded. Swollen gels were then removed from water and redried in the vacuum oven for 48 h before being weighed to measure the final dry weight ($W_d$). The swelling ratio was calculated by subtracting the dry weight ($W_d$) from the wet weight ($W_s$), then dividing by the final dry weight ($W_d$) [Eq. (4)]. Sol fraction was calculated taking the initial dry weight ($W_i$) minus the final dry weight ($W_d$) divided by the initial dry weight ($W_i$) [Eq. (5)]. Sample size of six hydrogels was tested for swelling ratio and sol fraction.

$$\textit{Swelling ratio} = \frac{W_s - W_d}{W_d} : \quad (4)$$

$$\textit{Sol Fraction} = \frac{W_i - W_d}{W_i} : \quad (5)$$

Hydrogel elasticity. Hydrogel samples of height to width of 2:1 (diameter = 5 mm) were fabricated for compression testing on an mechanical testing system (MTS) (Chatillon TCD 200; Wagner Instruments, Greenwich, CT, USA). Before testing, hydrogels were swollen in deionized (DI) water for 12 h in ambient conditions. Compression testing was run on an unclosed platform using flat platen with a crosshead speed of 5 mm/min. Load and displacement was recorded until failure, in which the Young’s modulus was determined by calculating the stress versus strain curves and determining the slope of the elastic region.

Cytotoxicity analysis
NIH/3T3 cells were purchased from ATCC (CRL-1658) and cultured in DMEM medium supplemented with 10% FBS and 1% penicillin–streptomycin and fungizone and maintained at 37°C with 5% carbon dioxide. Culture medium was changed every 2 days.

In vitro cytotoxicity was investigated using live/dead viability cytotoxicity kit. Cells were enzymatically removed (0.25% trypsin-EDTA), seeded in 24-well plates at 40,000 cells/cm². Following 24-h attachment and incubation, the cells were treated by adding 500 μL of 25% (wt%) hydrogel solutions dissolved in growth media with and without thermal crosslinking initiators (0.1 mM AA/APS) and co-polymer PEGDA (16.5% OPF and 8.5% PEGDA). After a 15 min incubation, 1 mL of additional media was added to each well. After an additional 24 h, cells were washed 3x with PBS and stained with dye solutions according to manufacturer’s instructions. In brief, 4 mM ethidium homodimer-1 and 2 mM calcein AM were reconstituted in growth media. Five hundred microliters of dye solution was added to each sample followed by a 45 min incubation. Cells were imaged using confocal microscopy (Olympus IX81; live: ex 488 nm/em BA505–525 nm, dead: ex 543 nm/em 560–660 nm) at a 40x magnification. Control samples consisted of living cells, with media changes during solution exchange procedures, and dead controls incubated with 70% ethanol for 30 min before assay.

Statistics

All groups were compared in GraphPad Prism 6 starting with a one-way analysis of variance with 95% confidence interval. If significance was found, then a two-way t-test was used to determine statistical differences between treatments. Sample sizes equaled three unless otherwise noted.

Experiment
**Physical characterization**

Nitrogen sparging technique exhibits visual and physical differences when compared to the TEA acid scavenger method. N\textsubscript{2}-OPF appeared to be whiter than TEA-OPF, comparable to the PEG (M\textsubscript{w} 4600) precursor (Figure 3-2). TEA-OPF had a light brown coloration to it even after post-processing washes and precipitations, indicative of trace amount of HCl salts within the polymer.\textsuperscript{1} After N\textsubscript{2}-OPF went through the final drying stage, it appeared to be clumped due to intermolecular forces, while TEA-OPF had a fine grain consistency because of salt contamination. Furthermore, it was observed that TEA-OPF dissolved into aqueous solutions easier than the N\textsubscript{2}-OPF.

**Increased oligomerization of OPF utilizing nitrogen sparging methods**

Proton NMR of TEA-OPF and N\textsubscript{2}-OPF schemes both show the formation of an ester bond at 4.33 ppm corresponding to the formation of PEG/fumarate oligomers. A shift in fumarate olefin signal from 7.87 ppm to 6.87 ppm also denotes attachment of FuCl to PEG (Figure 3-3).\textsuperscript{36} DSC revealed no significant difference between the T\textsubscript{m} and crystallinity of N\textsubscript{2}-OPF (60.7 – 0.4°C and 64.1% – 2.1%) and TEA-OPF (60.3 – 1.3°C and 58.3% – 3.4%; Table 3-1). However, the ratio of PEG hydroxyl end groups (3.01 ppm) to monomer units was lower in N2-OPF compared to TEA-OPF indicating increased oligomerization.

Utilizing size exclusion chromatography to determine N\textsubscript{2}-OPF molecular weight (M\textsubscript{w} = 17,271 – 3632) and degree of polymerization (X\textsubscript{n} = 3.9 – 0.8) confirmed significant increases in OPF oligomerization over TEA-OPF (M\textsubscript{w} = 10,891 – 1368, X\textsubscript{n} = 1.8 – 0.2; Table 3-1 and Figs. 3-4A and 3-5A, B).

Furthermore, N\textsubscript{2}-OPF had a significantly lower polydispersity index 1.3 – 0.06 compared to TEA-OPF 1.4 – 0.04, indicating a more homogeneous product (Table 3-1 and
**Figure 3-4A.** OPF product removed during both reactions shows the difference in rate of oligomerization during TEA-OPF and N\textsubscript{2}-OPF processing (Figure 3-5). However, fluorescent analysis of eluted TEA-OPF revealed a fluorescent molecule associated with the elution of TEA-OPF oligomers that is absent from N\textsubscript{2}-OPF (Figure 3-4B).

**Characteristics of crosslinked hydrogels**

Using the thermal initiator AA/APS, N\textsubscript{2}-OPF crosslinked by itself and had a significantly higher swell ratio than all other groups (Figure 3-6). However, when crosslinked in the presence of PEGDA, there was no difference in water up-take between TEA-OPF and N\textsubscript{2}-OPF hydrogels. Using UV initiation, N\textsubscript{2}-OPF with PEGDA had a significantly lower sol fraction than UV-initiated N\textsubscript{2}-OPF without PEGDA but no comparison could be made with TEA-OPF as it would not crosslink using the UV initiator even with the addition of PEGDA (Figure 3-6). Furthermore, TEA-OPF by itself did not crosslink with either UV or thermal crosslinking methods, while N\textsubscript{2}-OPF crosslinked under all conditions (Figure 3-6).

Compressive modulus of the OPF varied in range from 7.32 – 1.39 to 189.23 – 56.04 KPa, depending on the synthesis and crosslinking method, as well as presence of PEGDA linker (M\textsubscript{n} 575; Figure 3-7). TEA-OPF only cross-linked with the addition of PEGDA linkers and thermal crosslinking initiators. Following crosslinking, TEA-OPF’s modulus was 20.67 – 1.77 KPa while the N\textsubscript{2}-OPF hydrogels was 82.59 – 10.62 KPa, roughly a three-fold increase in compressive modulus. The presence of PEGDA also has the same trend of increasing stiffness. Photoinitiated and thermal initiated crosslinking methods with PEGDA caused photoinitiated hydrogels to have a higher modulus (189.23 – 56.04 KPa) than
thermally initiated hydrogels (82.59 – 10.62 KPa). Statistics indicated that all OPF hydrogel moduli were significantly different (p < 0.05).

Cell viability in the presence of polymer products

The influence of OPF oligomer products on cell growth and viability was examined by 24 h incubation of NIH/3T3 cells with different OPF hydrogel solutions. The cytotoxicity of all thermal initiator containing solutions was significant and resulted in low viability in culture. With the addition of PEGDA, little increase in viability was observed. Furthermore, PEGDA alone was shown to be acutely toxic to cells (Figure 3-8). To assess the cytotoxicity of individual OPF products, solutions containing 25 wt% N₂- and TEA-OPF in complete growth media were exposed to cells for 24 h. As a result of residual TEA-HCl salts, cells exposed to TEA-OPF alone were nonviable after 24 h, while cells exposed to N₂-OPF were over 90% viable in culture (Figure 3-8).

Discussion

The reaction of PEG and FuCl form linear polyester dimers, trimers, and eventually oligomers in a step-growth process. The primary limitation of this reaction is the production of HCl byproducts which must be isolated to prevent secondary reactions. Previously, removal of HCl was accomplished by acid scavengers such as TEA, potassium carbonate, or sodium hydroxide.\(^{16,23-28}\) In the current work, OPF synthesis using TEA was compared to an OPF synthesis using nitrogen sparging. The use of sparging for the in situ removal of HCl eliminates the need for an acid scavenger, thereby avoiding the production of difficult to remove TEA salts.

For both reaction methods, formation of PEG/fumarate oligomers was confirmed by \(^1\)H NMR. With greater olefin (6.87 ppm) to PEG (3.38–3.85 ppm) ratio and a reduction in
signal from PEG hydroxyl end-groups (3.01 ppm), N2-OPF resulted in a greater degree of oligomerization and larger molecular weight than TEA-OPF synthesis methods.\textsuperscript{1} Size exclusion chromatography confirms a significant increase in N2-OPF molecular weight and a more uniform product over TEA-OPF (\textbf{Table 3-1 and Figures 3-4A and 3-5A, B}).

In side-by-side comparison of TEA-OPF and N2-OPF reactions, temporal analysis of the product demonstrates a limited maximum achievable molecular weight using TEA-OPF methods. After 4 h, the maximum molecular weight was obtained by TEA-OPF with no additional oligomerization at subsequent time points. In contrast, the oligomerization of the N2-OPF method continues through 12 h (\textbf{Figure 3-5}). In step-growth processes, the final conversion, and hence the average molecular weight, is affected by the concentration of condensation byproducts.\textsuperscript{37} \textit{In situ} removal of acid byproducts during nitrogen sparging eliminates TEA salt formation allowing for the synthesis of a higher molecular weight OPF (\textbf{Figure 3-5A}).\textsuperscript{1,22}

In contrast to the nitrogen sparge method, TEA-OPF synthesis requires the additional steps of filtration, recrystallization, and solvent washing to remove acidic by-products.\textsuperscript{1,2} The incomplete removal of TEA salts during filtration, recrystallization, and solvent washing is apparent in \textsuperscript{1}H NMR (1.4 ppm). The TEA-OPF also retains a brown coloration even after repeated filtrations steps. Alternatively, the pure N2-OPF appears white immediately after synthesis and does not require additional purification steps. Fluorescent analysis of eluted product through GPC reveals the presence of a fluorescent molecule within TEA-OPF that is absent in N2-OPF (\textbf{Figure 3-4B}). The fluorescent signal corresponds with the elution time of TEA-OPF oligomers, indicating that the TEA salts are complexed to the OPF backbone. Cai and Wang have previously reported the formation of cell-cytotoxic TEA complexes during
polymerization of FuCl, in the presence of TEA, that cannot be completely removed during purification.\textsuperscript{22} This behavior is confirmed in the cytotoxicity analysis of OPF products.

To examine the crosslinking capabilities of N$_2$-OPF and TEA-OPF, two different commonly used free-radical initiator systems were applied; AA/APS, a thermally activated initiator, and Irgacure 2959, a UV activated initiator.\textsuperscript{1,2,20,21,38} In addition, hydrogels were fabricated with and without PEGDA, a copolymer commonly used in the crosslinking of PEG-based hydrogels due to the presence of highly reactive acrylic end groups. The use of PEGDA allows for more efficient network formation of resulting hydrogels.\textsuperscript{39,40}

Hydrogels were successfully crosslinked with AA/APS and PEGDA for both TEA-OPF and N$_2$-OPF. However, UV-initiated TEA-OPF/PEGDA and TEA-OPF hydrogels did not crosslink adequately to maintain their three-dimensional structure when placed in water, and subsequently fell apart. In comparison, N$_2$-OPF readily crosslinked using both initiator systems with and without PEGDA, highlighting the versatility of N$_2$-OPF product (Figures 3-6 and 3-7). The inefficient removal of TEA salts in the TEA-OPF synthesis method is likely a significant factor in the crosslinking behavior of TEA-OPF. The presence of TEA salts limits UV intensity and penetration into the gels. Also, the TEA salts may directly interfere with free-radical propagation and crosslinking reaction. To overcome the limitations of TEA-OPF, previous groups have increased reaction time to an hour to fully crosslink TEA-OPF.\textsuperscript{1,18,21} As OPF is commonly used as a biomaterial for cell encapsulation, minimizing UV exposure time reduces cellular cytotoxicity.\textsuperscript{20,21} Crosslinking of hydrogels was therefore limited to a 15 min exposure period, a time which has been shown to optimally balance the crosslinking reaction with cell viability.\textsuperscript{21} Fully crosslinked OPF hydrogels exhibited swelling characteristics dependent on the synthesis method and the use of the
crosslinking molecule. Using AA/APS and PEGDA as the crosslinker, there was no difference in swelling between TEA-OPF and N$_2$-OPF hydrogels. While TEA-OPF did not crosslink using the UV initiator, the choice of initiator did not influence the swelling of the N$_2$-OPF/PEGDA hydrogels. Furthermore, N$_2$-OPF was able to form hydrogels without the addition PEGDA, but resulted in a hydrogel with less crosslinking density and greater water uptake capability (Figure 3-6A).$^{17,41}$

All formulations were fabricated into gels of a 2:1 height to diameter ratio (diameter = 5 mm). As seen with the swell ratio study, the only TEA-OPF formulation that maintained its integrity was the TEA-OPF with PEGDA and the thermal initiator, so it was the only TEA-OPF formulation studied. When compared to thermally crosslinked TEA-OPF with PEGDA, N$_2$-OPF was found to be four times stronger than the TEA-OPF hydrogels (Fig. 3-7). This is due to the lack of crosslinking efficiency of the TEA-OPF, in part, to the reduced quantity of fumaryl groups in each TEA-OPF molecule. In addition, the presence of HCl salts in the TEA-OPF could be hindering the crosslinking efficiency, which explains the reduced amount of crosslinking, thus leading to a weaker modulus when compared to thermally crosslinked N$_2$-OPF with PEGDA.

The presence of PEGDA, a known linker for creating uniform polymer networks, increased the moduli of both thermal and photoinitiated N$_2$-OPF hydrogels.$^{42,43}$ As such, PEGDA allows for tunable compressive moduli ranging from 7 to 190 KPa. Interestingly, when N$_2$-OPF was cross-linked with PEGDA the modulus of UV crosslinking was increased over thermal crosslinking methods. However, in the absence of PEGDA thermal initiation method exhibited increased the modulus over UV methods. It is believed that the UV crosslinking is more efficient at radicalizing the acrylates in PEGDA, resulting in a higher
density of crosslinkable bonds, thus a stiffer material. The sol fraction of the N2-OPF with PEGDA supports that more of the hydrogel solutions has been crosslinked (Figure 3-6).

Since N2-OPF is the only product that can be crosslinked with and without PEGDA and utilize both light and heat activated free-radical initiators, N2-OPF can be fabricated into hydrogels with a wider range of mechanical properties, increasing its utility beyond that of TEA-OPF.

The importance of producing a polymer, which can be crosslinked without the addition of PEGDA, becomes even more apparent when cell cytotoxicity is analyzed. With the addition of PEGDA, significant cytotoxicity of NIH/3T3 cells was observed (Figure 3-8). Similar behavior has been observed in low molecular weight PEG methyl ether acrylate (Mn ~ 480) solutions, caused by a decline in the antioxidant levels of glutathione and increased intracellular reactive oxygen cytotoxicity. Furthermore, the exposure of cells to uncrosslinked N2-OPF showed little cytotoxicity, while TEA-OPF solutions showed significant cell death due to the persistence of cytotoxic HCl salts.

Conclusion

Through nitrogen sparging, we have shown that OPF can be produced with decreased postprocessing, increased product purity, greater oligomerization, and cell viability. These properties lead to greater tunability in mechanical properties and a more versatile hydrogel for biomedical applications. Instead of using TEA, an acid scavenger, in situ nitrogen gas sparging eliminates the need for post-processing washes using ethyl acetate and ethyl ether, which have proven to be inefficient while also being acutely toxic and potentially explosive through peroxide formation. With greater oligomerization, N2-OPF has more unsaturated
double bonds per molecule that can be used for crosslinking and addition of covalently bound side chains. This straightforward method of fabrication allows for facile production that eliminates postprocessing. The simplification of synthesis and elimination of impurities will expand the utility of N₂-OPF as a degradable hydrogel for cell culture, tissue engineering, regenerative medicine, and therapeutic delivery, among other applications.

**Acknowledgments**

The authors thank Dr. Shawn Dirk and Dr. Chester Simoko for their assistance with OPF synthesis and hydrogel characterization, respectively.

**Disclosure Statement**

No competing financial interests exist.

**Funding Information**

This work was supported by awards from the American Heart Association (10BGIA4570031), the National Science Foundation (CBET 1351947), NSF IGERT (0504276), NSFLS-AMP (HER1026412 [BDVIII]), NIH IMSD (5R25- GM060201), and NIH PREP (R25-GM075149). This work was performed, in part, at the Center for Integrated Nanotechnologies, an Office of Science User Facility operated for the U.S. Department of Energy (DOE), Office of Science by Los Alamos National Laboratory (Contract DE-AC52– 06NA25396), and Sandia National Laboratories (Contract DE-AC04-94AL85000).
Figure 3-1. Glassware set-up for the one-pot polycondensation of FuCl with PEG in DCM using (A) the acid scavenger TEA or (B) nitrogen sparging to remove HCl byproducts. (C) OPF reaction schemes for TEA-OPF and (D) (N₂-OPF). (E) Crosslinking structure of OPF oligomers through consumption of unsaturated double bonds. DCM, dichloromethane; FuCl, fumaryl chloride; HCl, hydrochloric acid; N₂-OPF, nitrogen-sparged OPF; OPF, oligo(poly(ethylene glycol) fumarate); PEG, polyethylene glycol; TEA, triethylamine.
Figure 3-2. Samples of (A) precursor poly(ethylene glycol) (PEG, 4,600 g/mole) polymer and OPF product using (B) triethylamine synthesis (TEA-OPF) and (C) nitrogen sparge synthesis (N₂-OPF).
Figure 3-3. $^1$H NMR spectra of 4.6 K OPF from (A) nitrogen sparging or (B) TEA scavenging methods. The presence of ester groups (b, 4.33 ppm) and olefin groups (c, 6.87 ppm) indicate bond formation and oligomerization of OPF. NMR (300 MHz) measurements were carried out in CDCl$_3$ (7.24 ppm). Some residual DCM can be observed at 5.26 ppm with TEA contaminants at 1.43 ppm. CDCl$_3$, deuterated chloroform; $^1$H NMR, hydrogen nuclear magnetic resonance.
Figure 3-4. (A) Representative elution profiles of OPF polymer products and pure 4.6 K PEG as determined by GPC. Decreased retention time indicates higher molecular weight N$_2$-OPF oligomers. (B) Fluorescence profiles of OPF products and 4.6 K PEG were collected in series with GPC. A large fluorescence peak was detected only in TEA-OPF indicating unremoved TEA-HCl, Ex 250 nm/Em 410 nm. GPC, gel permeation chromatography.
Figure 3-5. During synthesis, small samples of (A) N$_2$-OPF and (B) TEA-OPF product were removed periodically over 14 h and analyzed by GPC. Each labeled peak (i–iv) corresponds to an increasing oligomer length. TEA method limits molecular weight after addition of fumarate to reaction vessel (4 h). Color images are available online.
Figure 3-6. Swelling ratio of crosslinked OPF hydrogels using (A) thermal and (C) UV initiator methods. Sol Fraction of crosslinked OPF hydrogels using (B) thermal and (D) UV initiator methods. Swelling of N$_2$-OPF without PEGDA was significantly greater than all other gels, $p < 0.05$, $n = 6$. UV-initiated N$_2$-OPF with PEG-DA had significantly lower sol fraction than N$_2$-OPF without PEGDA, $p < 0.05$, $n = 6$. D indicates that gels did not crosslink sufficiently to be characterized. PEGDA, polyethylene glycol diacrylate; UV, ultraviolet.
Figure 3-7. Compression strength of OPF hydrogels with and without PEGDA linkers, and UV or thermally crosslinked. N₂-OPF hydrogels crosslinked with both UV and thermal initiators, with and without PEGDA linkers. TEA-OPF only crosslinked with thermal initiation with PEGDA linkers. All hydrogel types had significantly different moduli, \( p < 0.05, n = 8 \). N₂-OPF with PEGDA hydrogels had a significantly stronger modulus, \( p < 0.05, n = 8 \).
Figure 3-8. Cell viability and cytotoxicity staining of NIH/3T3 (A) living controls compared to cells exposed to (B) N$_2$-OPF, (C) PEGDA, and (D) TEA-OPF solutions. TEA-OPF as well as the required secondary crosslinker PEGDA shows acute toxicity, while N$_2$-OPF shows no toxicity over the live cell controls. Images are colocalized (green = live, red = dead, Scale 50 mm). Color images are available online.
### Tables

**Table 3-1. Material Properties of Triethylamine-Oligo(Poly(ethylene glycol) Fumarate) and Nitrogen-Sparged Oligo(Poly(ethylene glycol) Fumarate)**

<table>
<thead>
<tr>
<th>Synthesis Method</th>
<th>Percentage of Yield</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
<th>Percentage of Crystallinity (X)</th>
<th>M&lt;sub&gt;n&lt;/sub&gt; (g/mol)</th>
<th>M&lt;sub&gt;w&lt;/sub&gt; (g/mol)</th>
<th>PDI</th>
<th>X&lt;sub&gt;n&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEA-OPF</td>
<td>86.8±9.2</td>
<td>60.7±0.4</td>
<td>64.1±2.1</td>
<td>7779±847</td>
<td>10,891±1368</td>
<td>1.4±0.04</td>
<td>1.8±0.2</td>
</tr>
<tr>
<td>N&lt;sub&gt;2&lt;/sub&gt;-OPF</td>
<td>95.1±3.0</td>
<td>60.3±1.3</td>
<td>58.3±3.4</td>
<td>13,097±3494</td>
<td>17,271±3632*</td>
<td>1.3±0.06*</td>
<td>3.93±0.8*</td>
</tr>
</tbody>
</table>

All OPF synthesized with 4.6 K PEG.

M<sub>n</sub>, number average molecular weight; M<sub>w</sub>, weight average molecular weight; N<sub>2</sub>-OPF, nitrogen-sparged OPF; OPF, oligo(poly(ethylene glycol) fumarate); PDI, polydispersity index; PEG, polyethylene glycol; TEA, triethylamine; X<sub>n</sub>, degree of polymerization.

*p < 0.05, n = 3–4.*
References


poly(propylene fumarate)/poly(DL- lactic-co-glycolic acid) composite scaffolds. Biomaterials 26, 3215, 2005.


Received: February 3, 2021
Accepted: March 18, 2021
Online Publication Date: May 18, 2021
Supplemental

This section describes work of CTD that contributed to the published manuscript presented above.

Cytotoxicity of nitrogen sparged OPF (N₂-OPF) and triethylamine OPF (TEA-OPF)

Preliminary cell work run by C.T.D. to study the cytotoxicity of the N₂-OPF to TEA-OPF at varying weight percent concentrations to answer if the concentration of each OPF polymer affects the viability of 3T3 cells and determine if N₂-OPF is less cytotoxic than TEA-OPF.

Materials and Methods

Reagents and Chemicals

Culture reagents Dulbecco’s minimum essential growth medium (DMEM), fetal bovine serum (FBS), Dulbecco’s phosphate-buffered saline (PBS), penicillin–streptomycin (Pen-Strep), fungizone (Fungi), trypsin-ethylenediaminetetraacetic acid 0.25% (Trypsin-EDTA), and live/dead cell viability kit (Molecular Probes) were purchased from ThermoFisher Scientific.

Condition Media Preparation

The conditioned media were prepared by dissolving various concentrations of N₂-OPF and TEA-OPF in primary media. The concentration of the OPFs in media ranged from 0.25%, 2.5%, 10%, and 25% (w/v) (Table S3-1). Briefly, a 25% (w/v) solution was made by adding 500 mg of either N₂-OPF or TEA-OPF into 1.5 mL of DMEM media. The 25% (w/v) solution was then diluted down to the corresponding dilutions (10%, 2.5%, 0.25%) through the addition of more media. The prepared solutions were filtered using a cellulose acetate membrane filter (0.2 m pore diameter) for sterilization prior to administering to cells.
Cytotoxicity of \(N_2\)-OPF and TEA-OPF

NIH/3T3 cells were purchased from ATCC (CRL-1658) and cultured on a T-75 culture flask in DMEM medium supplemented with 10% FBS, 1% Pen-Strep, and 1% fungizone and maintained at 37°C with 5% carbon dioxide. The culture medium was changed every two days. At 80% confluences cells were enzymatically lifted by exposure to a trypsin-EDTA solution (2 mL/flask). Cells were seeded onto 48-well tissue culture plates in media at 32,000 cells/well and allowed to attach for 24-hours at culture conditions (37°C, 95% RH, 5% \(CO_2\)). After 24-hours the media was aspirated and three wells of attached 3T3s were exposed to 200 μL of the prepared conditioned media treatment and incubated for 2-hours and 24-hours. Treatment solutions were aspirated, and cells were washed with 200 μL of DPBS to remove any remaining conditioned media. For a positive (live) control and negative (dead) control, 3T3s were cultured in media and treated with 70% (v/v) ethanol solution for 30-minutes. LIVE/DEAD reagent [combination of 4 M ethidium homodimer-1 (EthD-1) and 2 M calcein AM] was prepared following the protocol provided by the manufacturer and 100 μL of the LIVE/DEAD reagent was added to each sample well and incubated in the dark for 30-minutes at room temperature. Cells were then viewed on an inverted microscope with a fluorescence attachment and microscope filter cubes for calcein AM (Live, Green, 494/517 nm) and EthD-1 (Dead, Red, 528/617 nm).

Fluorescent image processing was performed with Fiji (NIH/ImageJ). Briefly, an image was split into its corresponding red, green, and blue channels. The blue channel was removed because there was no blue fluorescent stain used and the red and green channels were then merged into one image. Finally, to reduce the background noise and focus on the
intensity of the fluorescent data, the brightness and/or contrast was adjusted to incorporate only signals associated with the live or dead stain of a cell.

Results

Initially, 3T3 fibroblasts were exposed to N$_2$-OPF or TEA-OPF conditioned media for 24-hours. The lower concentrations of 0.25% and 2.5% (w/v) for both polymer solutions resulted in adhered cells, spindled morphology, and high viability (Figure S3-1). Low amounts of cytotoxicity were observed for the 10% (w/v) condition media for both polymers. Complete cell death was observed for 25% (w/v) N$_2$-OPF and TEA-OPF condition media. Biocompatibility differences between the two polymers were not found and both polymer solutions followed the same trend of increased cytotoxicity as polymer concentration increased.

Further investigation into the 10% and 25% (w/v) concentrations were run by exposing 3T3 cells to N$_2$-OPF and TEA-OPF conditioned media for 2-hours, then analyzed for cell viability. Like the 24-hour test, both N$_2$-OPF and TEA-OPF at a concentration of 25% (w/v) had cytotoxic effects on 3T3 fibroblast (Figure S3-2). The 10% (w/v) concentration had varying results. Cells exposed to the N$_2$-OPF solution showed higher cytotoxicity, compared to the TEA-OPF treatment.
Table S3-1. N<sub>2</sub>-OPF and TEA-OPF treatments at varying concentrations and the formulations to make each conditioned media treatment.

<table>
<thead>
<tr>
<th>Treatments (w/v)</th>
<th>Formulation and Dilution of Treatments</th>
<th>Solution Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>25% N&lt;sub&gt;2&lt;/sub&gt;-OPF</td>
<td>500 mg N&lt;sub&gt;2&lt;/sub&gt;-OPF into 1.5 mL DMEM media</td>
<td>N&lt;sub&gt;2&lt;/sub&gt;-OPF Stock</td>
</tr>
<tr>
<td>10% N&lt;sub&gt;2&lt;/sub&gt;-OPF</td>
<td>400 μL of N&lt;sub&gt;2&lt;/sub&gt; Stock into 600 μL DMEM media</td>
<td>1 mL</td>
</tr>
<tr>
<td>2.5% N&lt;sub&gt;2&lt;/sub&gt;-OPF</td>
<td>100 μL of N&lt;sub&gt;2&lt;/sub&gt; Stock into 900 μL DMEM media</td>
<td>1 mL</td>
</tr>
<tr>
<td>0.25% N&lt;sub&gt;2&lt;/sub&gt;-OPF</td>
<td>10 μL of N&lt;sub&gt;2&lt;/sub&gt; Stock into 990 μL DMEM media</td>
<td>1 mL</td>
</tr>
<tr>
<td>25% TEA-OPF</td>
<td>500 mg TEA-OPF into 1.5 mL DMEM media</td>
<td>TEA-OPF Stock</td>
</tr>
<tr>
<td>10% TEA-OPF</td>
<td>400 μL of TEA-OPF into 600 μL DMEM media</td>
<td>1 mL</td>
</tr>
<tr>
<td>2.5% TEA-OPF</td>
<td>100 μL of TEA-OPF into 900 μL DMEM media</td>
<td>1 mL</td>
</tr>
<tr>
<td>0.25% TEA-OPF</td>
<td>10 μL of TEA-OPF into 990 μL DMEM media</td>
<td>1 mL</td>
</tr>
</tbody>
</table>
Figure S3-1. Cytotoxicity analysis of 3T3 fibroblasts exposed to N₂-OPF (A-D) or TEA-OPF (E-H) conditioned media for 24-hours with solution concentrations of 0.25% (A, E), 2.5% (B, F), 10% (C, G), 25% (D, H). Live (I) and dead (J) controls. Live = Green, Dead = Red, Scale = 100 μm
Figure S3-2. 3T3 fibroblasts exposed to N$_2$-OPF (A, B) or TEA-OPF (C, D) conditioned media for 2-hours. Concentrations of 10% (A, E) and 25% (B, D) were tested for further cytotoxic effects. Live (E) and dead (F) controls. Live = Green, Dead = Red, Scale = 100 μm
Chapter 4

Charged Oligo(Poly(Ethylene Glycol) Fumarate) Hydrogels for

Encapsulating Valvular Interstitial Cells

Christian T. Denny\textsuperscript{1,2} and Elizabeth L. Hedberg-Dirk, PhD\textsuperscript{1,2,3}

\textsuperscript{1}Center for Biomedical Engineering, University of New Mexico, Albuquerque, New Mexico, USA.

\textsuperscript{2}Biomedical Engineering Graduate Program, University of New Mexico, Albuquerque, New Mexico, USA.

\textsuperscript{3}Department of Chemical and Biological Engineering, University of New Mexico, Albuquerque, New Mexico, USA.
Abstract

Cardiovascular disease is the number one cause of death in the United States. Calcific aortic valvular disease (CAVD) is the leading contributor to cardiovascular disease and is characterized by thickening and stiffening of the aortic valve leaflets through fibrotic and calcific nodule build-up. The resident cell population found in the heart valve leaflets are valvular interstitial cells (VIC). Healthy aortic valve leaflets are maintained by quiescent (qVIC) and activated VIC (aVIC) phenotypes but, through environmental factors that are not well understood, VICs can differentiate into calcific nodule forming osteoblastic-like VICs (obVIC). Recently, it was found the positively charged surfaces cause VICs to differentiate into obVICs and produce calcific nodules. Therefore, this work aims to investigate the effects of charged three-dimensional (3D) environments on VIC phenotype. It is hypothesized that positively charged environments will stimulate VICs to become obVICs. To design a 3D cell culture platform oligo(poly(ethylene glycol) fumarate) (OPF), a biodegradable polymer containing unsaturated double bonds, and a cell attachment biomolecule were synthesized and combined with poly(ethylene glycol) diacrylate (PEGDA) cross-linker to form hydrogels. The positively charged monomer [2-(Methacryloyloxy)ethyl] trimethylammonium chloride (MAETAC) was then incorporated into the hydrogel network for testing environmental charge effects on VICs. It was found that concentrations up to 200 mM of MAETAC could be incorporated into the OPF and PEGDA hydrogel network. It was also found that VIC populations were not supported by the OPF and PEGDA hydrogels due to the pH of the OPF and PEGDA molecular weight (Mₙ 575). Finally, a more water-soluble photoinitiator was investigated for cross-linking speed and found to decrease cross-linking time which decreased the amount of UV (365 nm) light VICs were exposed to during the
encapsulation process. It was concluded that although the current OPF and PEGDA hydrogel platform did not support VIC growth, further investigation into neutralizing the OPF’s and increasing the PEGDA molecular weight may improve VIC survival through encapsulation and in culture.
1. Introduction

With 859,125 annual deaths, cardiovascular disease is the number one cause of death in the United States. Globally it accounts for 17.8 million annual deaths.\textsuperscript{1,2} Calcific aortic valvular disease (CAVD) is a leading cause of cardiovascular disease. This disease is characterized by increased thickness and reduced flexibility of the aortic valve.\textsuperscript{1–3} The rigidity of the valve restricts the normal motion of the valve and disrupts the coaptation of the valve leaflets, causing stenosis (\textbf{Figure 4-1}). Stenosis leads to regurgitation of oxygenated blood back into the heart ventricle subsequently lowering blood pressure resulting in poor circulation to the body and heart.\textsuperscript{4} Significantly, poor circulation to the coronary arteries results in a negative feedback loop where insufficient amounts of oxygenated blood is delivered to the heart. These conditions force the heart to work harder to pump more blood to the body producing more stress on the aortic valve, further exacerbating CAVD and ultimately leading to heart failure if left untreated.

Healthy leaflets are maintained by a heterogeneous cell population called valvular interstitial cells (VIC). The most common phenotypes found throughout the leaflet are quiescent (qVIC) which are characterized by a cobbled morphology \textit{in vitro} (\textbf{Figure 4-2}). During normal valve activity qVICs maintain valvular structure and stability through low extracellular matrix (ECM) turnover. In response to inflammatory factors and cytokines, qVICs differentiate into a highly proliferative, activated VIC (aVIC) phenotype to help repair the damaged leaflet. Activated VICs are characterized by an elongated-spindle morphology and expression of \(\alpha\)-smooth muscle actin (\textbf{Figure 4-2}). Normally, after the damage has been repaired and inflammation has reduced, aVICs undergo apoptosis or revert to qVICs. If aVICs remain, extensive remodeling and disorganization of the heart valve leaflet occurs and
leads to fibrosis. Over time, the fibrotic ECM stiffens the valve leaflet contributing to the differentiation of aVICs into an osteoblastic-like (obVIC) phenotype. Osteoblastic-like VICs are characterized by an irregular morphology and expression of osteoblastic markers such as osteocalcin and transforming growth factor β-1. Most notably, obVICs produce calcific nodules in the ECM of the leaflet which, in conjunction with the buildup of fibrotic tissue, promotes nearby VICs to switch to obVICs further propagating the disease throughout the valve (Figure 4-2).

Despite its prevalence, the factors that cause VICs to become osteoblastic-like remain unclear. Environmental factors such as material properties, biological cues, and soluble factors all have been shown to alter VIC phenotype. Specifically, material properties such as substrate stiffness and chemistry have been used to direct VIC phenotype. When VICs are grown on tissue culture polystyrene (TCPS), a gold standard for tissue culture, the stiff plastic (30.6 ± 10.9 GPa) support aVICs. The aVICs proliferate quickly until they become fully confluent at which time they differentiate into obVICs and start producing calcific nodules. In comparison, the valve has been reported to have a modulus of 25 kPa, considerably less stiff than the TCPS. Evidence based on substrate stiffness and CAVD valve stiffening suggest that mechanical factors may induce VICs to become specific phenotypes. Furthermore, substrate chemistry is consistently used to improve cell attachment and control cell phenotype. Tissue culture polystyrene undergoes plasma treatment which oxygenates the ring of polystyrene. This imparts a negative charge and increases cell attachment and viability. Additionally, we previously studied how charged surfaces affect VIC phenotype by seeding VICs on top of charged self-assembled monolayers made from alkanethiols. These monolayers either presented a positive (NH₃⁺) or a negative (COO⁻) charge to one side of
seeded VICs. It was found that over 7-days VICs exhibited surface-dependent differences in morphology and osteoblastic potential. Positively charged surfaces had the most obVICs and calcific nodule formation. The negatively charged surfaces showed majority aVICs and no calcification.\(^2^0\) Based on these findings, material properties are of interest to design microenvironments for studying CAVD development, drug development, and improving current treatments.

Due to the lack of physiologically relevant laboratory models and recent advances in valvular tissue engineering there has been an aim to create three-dimensional (3D) microenvironments that represent the valve milieu. Within the tissue engineering field, polymer networks such as hydrogels that are highly hydroscopic have been used extensively to create soft tissue. One such polymer, poly(ethylene glycol) (PEG), is a popular synthetic polymer that is extensively used. Poly(ethylene glycol)’s biocompatibility, hydrophilicity, and bioinert properties have made it useful for creating and systematically controlling microenvironments for tissue engineering applications.\(^2^1\)–\(^2^3\) Specifically, PEG dendrimers have been combined with biodegradable and cell attachment peptides to improve and study degradation rate on VICs in 3D cell culture.\(^2^4\)–\(^3^0\) Moreover, linear PEG has been functionalized with \([2-(\text{methacryloyloxy})\text{ethyl}]\text{trimethylammonium chloride (MAETAC) to create positively charged substrates for growing cells.}\(^1^5\)–\(^1^9,3^1\)

In addition to PEG’s individual utility, PEG has been used to create co-polymers with a variety of properties for 3D cell culture. One such co-polymer made from PEG is oligo(poly(ethylene glycol) fumarate) (OPF) a biodegradable, linear polyester that contains multiple unsaturated fumarate bonds in its backbone that are useful for functionalization with moieties and cross-linking the polymers into a hydrogel (Figure 4-3). Since OPF is a PEG
based polymer, cell attachment is poor. To mediate this problem, tethered RGD cell attachment groups have been cross-linked into OPF hydrogels to support cell growth.\textsuperscript{32-34} Also, chemically charged end groups, like MAETAC, have been used to make charged OPF hydrogels for cell studies.\textsuperscript{9,10,12,35} This makes OPF ideal for systematically creating micro-environments to investigate how material properties, like substrate chemistry, affect VICs behavior.

Currently, there is little research on VICs behavior to charged substrates. Through our previous work with charged self-assembled monolayers we found that positively charged surfaces direct VICs to differentiate into obVICs and form calcific nodules. We hypothesize the same phenomenon will occur when VICs are scaled up and grown in a positively charged 3D culture platform. The objective of this work is to fabricate a positively charged OPF-based hydrogel, with tunable charge densities for testing VICs response to positively charged 3D micro-environments. To build the hydrogel platform, OPF and PEGDA cross-linker were used to form the base hydrogel network. Cell attachment peptide RGD and positively charged MAETAC were photo cross-linked into the hydrogels during VIC encapsulation. Then VIC cytotoxicity to the charged hydrogels components, in 2D culture studies, and within the OPF/PEG hydrogels were tested. Finally, a quicker photo-initiator was tested to improve cross-linking speed of the OPF/PEG hydrogel for the encapsulation of VICs process.

2. Materials and Methods

2.1 Synthesis of Oligo(Poly(Ethylene Glycol) Fumarate)

Oligo(poly(ethylene glycol) fumarate) was synthesized as outlined in Rush et al.\textsuperscript{36} Briefly, prior to running of the reaction, fumaryl chloride (FuCl, TCI, F0152) was distilled
and poly(ethylene glycol) (PEG 1,000 Da, Sigma-Aldrich, 807488) was azeotropically dried with toluene (Sigma-Aldrich, 244511). Fifty grams of PEG (1,000 Da) was dissolved in dichloromethane (DCM, VWR, BDH1113) in a 3-neck round bottom flask. The reaction was maintained under a nitrogen blanket to impede oxygen inhibition of the polymerization. The PEG solution was stirred vigorously while FuCl (0.9:1 molar ratio FuCl:PEG) was added dropwise over 4-6 hours to the reaction. Throughout the reaction, removal of hydrochloric acid (HCl) byproduct was accomplished by nitrogen (N$_2$) gas sparging using a fine fritted gas dispersion tube (Chemglass, CG-203-01) at a flow rate of 2.0 SCFH. The removed HCl was neutralized in a 1 M NaOH (VWR, 221465) in ethanol (Koptec, V1001). The reaction was allowed to progress for 48-hours under continual sparging with periodic DCM refills. At the completion of the reaction, the solvent was removed via rotary evaporation (Buchi, R-215).

2.2 Synthesis of Acrylate-Poly(Ethylene Glycol)-CGRGDS Biomolecule

To create the acrylate-poly(ethylene glycol)-CGRGDS (PEG-RGD) biomolecule, acrylate-poly(ethylene glycol)-succinimide (M$_n$ 3400, Laysan, SVA-3400) and CGRGDS (Sigma-Aldrich) were dissolved together in a 50 mM sodium bicarbonate buffer solution (pH 8.5). The reaction progressed for 2 hours at room temperature under a nitrogen environment at room temperature with vigorous stirring. Upon completion of the reaction, the solution was transferred into dialysis tubing (Spectrum Labs, 132108) with a pore size of 2,000 Da and dialyzed in ultra-pure water with periodic bath changes for 24-hours. The final PEG-RGD was freeze dried and stored under nitrogen at -20°C.

2.3 Proton-Nuclear Magnetic Resonance Spectroscopy

Proton nuclear magnetic resonance ($^1$H-NMR) spectroscopy was used to assess chemical structure of the OPF and the PEG-RGD biomolecule with an Avance III Solution
300 (300 MHz, Bruker, Massachusetts). Samples were prepared by dissolving 50 mg of OPF into 1 mL of deuterated chloroform (Millipore Sigma, Germany) while 50 mg of PEG-RGD was dissolved in 1 mL of deuterium oxide (Cambridge Isotope Lab, 7789-20-0). Samples were loaded into borosilicate NMR tubes (5 mm diameter, Wilmad, New Jersey) and scanned 16 times. Spectra were first processed by calibrating the baseline through the solvent peaks, deuterated chloroform at 7.26 ppm and deuterium oxide at 4.8 ppm, then sample peaks were identified and used to determine chemical structure.

2.4 Gel-Permeation Chromatography

The molecular weight and polydispersity index of OPF was measured using a Waters HPLC with a PLgel Mixed-C column (5 μm, Agilent Technologies, PL1110-6500) equipped with a refractive index detector (Waters, 1515, Waters 2414). The solvent used in the HPLC system was chloroform supplemented with 0.5% triethylamine. OPF was prepared in chloroform at a concentration of 5 mg/mL of solvent and filtered through a 13 mm syringe filter (VWR 28145-493), prior to the sample solution being loaded into a 1.5 mL vial containing a rubber injection lid. Two standards were created using a range of known poly(ethylene glycol) (Agilent Technology, PL2070-0100) weights. Standard A contained the following molecular weights (106, 410, 1,020, 3,870, 18,100 g/mole) and standard B contained the following molecular weights (194, 615, 1,450, 8,160, 21,160 g/mole). A calibration curve was created based on both standards and used to compare the OPF samples. The standards and samples were run at a flow rate of 1.0 mL/minute with a triplicate sample size.

2.5 Fabrication of Oligo(Poly(Ethylene Glycol) Fumarate) Hydrogels

The base OPF and poly(ethylene glycol) diacrylate (PEGDA, Mn 575) hydrogel was made with the following recipe as outlined in (Table 4-1). Briefly, OPF and PEGDA were
mixed in a final PBS solution at a 5:1 weight ratio (OPF: 20.83 wt%, PEGDA: 4.17 wt%). [2-(Methacryloyloxy)ethyl]trimethylammonium chloride (MAETAC, Sigma-Aldrich, 408107) was added to the OPF/PEG hydrogels at 50 mM, 100 mM, 200 mM, 400 mM concentrations to create the positively charged hydrogels for the sol-swell analysis. PEG-RGD biomolecule was added to the OPF/PEG solutions at concentrations of 1 mM and 2 mM for VIC encapsulation studies. Positively charged hydrogels used for the sol-swell and the PEG/PEGDA hydrogels used for encapsulation tests were cross-linked with the photoinitiator Irgacure 2959 (0.05 wt%, Sigma-Aldrich, 410896) in a UV Crosslinker box (Analytik Jena, CL-1000) for 15 minutes (365 nm, 10 mJ/cm²).

2.6 Sol Fraction & Swelling Ratio of Charged Hydrogels & LAP Cross-Linked Hydrogels

Two photo initiators, I2959 and LAP, were tested to determine the efficiency of crosslinking charged hydrogels. After cross-linking the hydrogels, they were weighed to obtain the weight initial (W_i) and placed into 10 mL of phosphate buffer saline (PBS) overnight. Swollen hydrogels were then weighed to obtain a swollen weight (W_s). Finally, hydrogels were dried in a vacuum chamber for 48-hours and reweighed to obtain the weight dried (W_d).

\[
\text{Swelling Degree} = \frac{W_s - W_d}{W_s} \quad [1]
\]

\[
\text{Sol Fraction} = \frac{W_i - W_d}{W_i} \quad [2]
\]

The following method was used for analyzing both the charged hydrogels and LAP cross-linked hydrogels.

2.7 Cytotoxicity of Encapsulated Valvular Interstitial Cells

VICs were obtained from porcine aortic valves (Hormel) through a collagenase digest procedure and stored in liquid nitrogen. VICs were passaged in a tissue culture polystyrene
(TSPS) flask with Media 199 (Hyclone, SH30253.01) containing 10% fetal bovine serum (ThermoFisher, 10438034), 1% penicillin/streptomycin (ThermoFisher, 15140122), and 1% amphotericin B (ThermoFisher, 15290026) at 5% CO₂, 37°C, and 90% humidity. After 2-3 passages, cells were frozen down and stored in liquid nitrogen. After thawing, cells were passaged 2-3 times before use.

To prepare cells for encapsulation, VICs were removed from TCPS surfaces through a trypsin/EDTA digest (ThermFisher, 25200072) and suspended in a OPF/PEG solution, sterile filtered using 0.22 μm syringe filter, at a concentration of 10x10⁶ cells/mL.²⁴,³⁸–⁴² The cell-laden hydrogel solution was then pipetted into Teflon molds with wells 6 mm diameter x 1 mm height and UV crosslinked for 15 minutes (365 nm, 10 mJ/cm²). Hydrogels were then removed from molds and placed into 48-well TCPS plate (Corning Inc, 3548) with 1mL of Media 199. Subsequently, the well plate was placed on an orbital shaker table and agitated in an incubator (37°C, 5%, CO₂) for 24-hours.

Live/Dead fluorescent analysis (calcein AM, ethidium homodimer-1, Molecular Probes-Invitrogen, C3100MP) was run to determine cell viability. Briefly, 1 mL of the staining solution was administered to each hydrogel construct and allowed to incubate for 30-minutes (room temperature, dark). A live control containing just cells seeded on the well plate and a dead control of cell laden hydrogels exposed to 70% ethanol for 30 minutes was used. Cells were then viewed on an inverted microscope with a fluorescence attachment where red stained cells indicated cell death and green stained cells signified live cells. Brightfield and fluorescent images were taken of the cell laden constructs and cells were visually analyzed to determine if VICs survived the encapsulation process with OPF/PEG and PEG-RGD biomolecules.

2.8 pH of Oligo(poly(ethylene glycol) fumarate)
OPF was dissolved in PBS (pH 7.4) at a 20.83 wt% representative of the OPF concentration used to form the hydrogels. The solution was mixed for 1-hour in the dark at which the pH (VWR, SB70P) of the OPF solution was measured.

2.9 Cross-Linking Kinetics of LAP Photo-Initiator

In this study, effects of LAP concentration, UV intensity, and cross-linking time was examined for faster crosslinking efficiency when compared to the currently used I2959 (15-minutes UV exposure time at 0.05 wt%). LAP was tested at 0.03, 0.05, and 0.075 wt% (Table 4-2). Additionally, UV intensities of 2, 5, and 10 mW/cm² were used in a UV Crosslinker box. The hydrogels were cast in syringe molds and cross-linked for 1.0, 1.5, or 3.0 minutes. The final hydrogels were analyzed through the sol fraction and swelling ratio analysis as previously outlined above.

2.10 Cytotoxicity of Leachable Components & the Hydrogel Solution

To determine the cytotoxicity of the materials leached products from the OPF/PEG hydrogel, the OPF/PEG hydrogel solution, and the individual polymer components were exposed to cells. A condition media with the leached components from the OPF/PEG hydrogels was prepared by soaking a hydrogel scaffold (1 mm by 6 mm, height to diameter) in 1 mL of VIC media for 24-hours. The conditioned media, the OPF/PEG hydrogel solution, a solution of OPF (20.83 wt%) in PBS, and a solution of PEGDA (4.17 wt%) in PBS were sterile filtered prior to application to cells.

VICs were seeded in a 48-well plate at a seeding density of 95,000 cells/well. Along with the conditioned media and OPF/PEG hydrogel solution, 100 μL of each individual component solution, OPF and PEGDA, were administered to the VICs. After 20-minutes the treatment solutions were removed and the VICs washed carefully with 200 μL of PBS.
Finally, the Live/Dead stains were administered and the VICs were imaged under an inverted microscope with a fluorescent source attached.

2.11 Statistics

A Shapiro-Wilks and Levene’s tests were used to determine the normality and equal variance within and between groups. A Kruskal-Wallis H analysis was used to determine statistical differences between multiple groups. The Kruskal-Wallis H Test is a rank-based nonparametric analysis that measures the statistical differences between two or more independent variables. If a statistical difference was found, Dunn’s multiple comparisons post-hoc test with a Bonferroni correction at a 95% confidence interval (α<0.05) was used to determine which group(s) were significantly different from each other. The Dunn’s test is a nonparametric multiple comparisons test and the Bonferroni correction was used to reduce error rates amongst multiple comparisons. All error bars are calculated based on mean ± standard deviation and statistical analyses were performed using Microsoft Excel and RStudio. Fluorescent images were analyzed using the Fiji software a version of NIH ImageJ software.

3. Results

3.1 Oligo(Poly(Ethylene Glycol) Fumarate) and Poly(Ethylene Glycol)-RGD

Characterization

OPF was synthesized in three 50-gram batches and characterized through \(^1\)H-NMR and GPC. The NMR spectrum showed characteristic olefin (6.8-7.0 ppm), PEG (3.4-4.0 ppm), and ester (4.3-4.5 ppm) peaks distinct to OPF. Confirming that the polymer has been synthesized (Figure 4-4).\(^{10}\) The GPC analysis showed that OPF had a weight-average molecular weight (\(M_w\)) of 8036 ± 115 g/mole and a number-average molecular weight (\(M_n\))
of $5,287 \pm 516$ g/mole with a PDI of $1.52 \pm 0.133$ (n=3). Calculations based on OPF’s $M_w$ indicated that on average there were $7 \pm 0.1$ double bonds per OPF molecule (Table 4-1).

Visual observations showed that OPF is a white powder that partially clumped together. The visual characteristics of OPF are similar to that of the PEG 1000 g/mole precursor monomer. Moreover, due to the PEG in the backbone, OPF readily dissolved into an aqueous solution.

During the formation of acrylate-PEG-RGD, the succinimide group on the end of the PEG chain is being replaced by the CGRGDS peptide and removed through dialysis. NMR comparison of the acrylate-PEG-succinimide ($M_n$ 3400) molecule to the synthesized PEG-RGD biomolecule showed that succinimide peaks (2.7-2.9 ppm) were present in the initial acr-PEG-succinimide molecule and had disappeared in the final biomolecule spectrum (Figure 4-5). Additionally, the PEG-RGD spectrum retained the acrylate (5.8-6.9 ppm) and PEG (1.4-1.8, 2.5-2.7, 3.2-4.0, 4.1-4.4 ppm) peaks found in the original acrylate-PEG-succinimide molecule. The spectrum also showed peptide peaks indicating that the CGRGDS had been immobilized onto the polymer and not removed during dialysis (Figure 4-5). Visual inspection of the final PEG-RGD biomolecule showed a white powder that had small grains and easily went into an aqueous solution.

3.2 Hydrogel Formation of Positively Charged Hydrogels

To measure how increasing MAETAC concentrations affected the formation of the base OPF/PEG hydrogel (OPF 20.83 wt%, PEGDA 4.17 wt%, I2959 0.1 wt%) physical properties, an analysis of the hydrogel’s swelling ratio and sol fraction was run with MAETAC concentrations 0 mM, 50 mM, 100 mM, and 400 mM. Only the 400 mM group had a swelling ratio that was significantly different from the 100 mM treatment (p-value = 0.0433) (Figure 4-6, TOP). Visual observations of the 400 mM MAETAC hydrogels
revealed that they did not retain their casted shape when handle and were susceptible to falling apart.

The sol fraction was used to determine how much of the OPF/PEG hydrogel solution was not cross-linked into the polymer network. The 400 mM concentration was found to be significantly different from the 50 mM and OPF/PEG control (p-values=0.00927, 7.04x10⁻⁵). The control (OPF/PEG), 50 mM, and 100 mM concentrations had on average a sol fraction between 50-60%, while the sol fraction was >60% for the 400 mM concentration (Figure 4-6, BOTTOM) which signifies that more gel solution is failing to cross-link into a polymer network as the MAETAC concentration increased.

3.3 Cytotoxicity of Encapsulated Valvular Interstitial Cells

Viability tests to determine if VICs survived the encapsulation process were performed with the control (OPF/PEG) hydrogels and compared to hydrogels containing 1mM or 2 mM PEG-RGD biomolecule. Initial observations show that VICs are dead in all groups. It was also found that within the cross-linked hydrogels there were aggregates of VICs leading to uneven distribution of cells throughout the hydrogel with all test groups (Figure 4-7). Furthermore, VICs displayed a rounded morphology despite the presence of cell attachment peptides. Therefore, it was concluded that something other than PEG-RGD concentration was causing VICs to not survive encapsulation. Since the OPF/PEG hydrogels with 1 mM and 2 mM PEG-RGD resulted in complete cell death, positively charged hydrogels were not tested for VIC survival during encapsulation. Further experiments explored what could be contributing to the hostile conditions leading to the VIC death.

3.4 pH of Oligo(poly(ethylene glycol) fumarate)
During the OPF polycondensation reaction hydrochloric acid is generated as a byproduct. Although a majority of the HCl is removed during the reaction, residual amounts of HCl could remain in the OPF and be the cause of the high mortality of VICs during the encapsulation. To investigate this hypothesis, OPF was added to PBS (pH 7.4) at the concentration (20.83 wt%) used for encapsulating VICs. The pH of the PBS solution dropped from 7.4 to 3.7, indicating that the OPF still contained considerable amounts of acidic contamination. Therefore, to mediate the extreme acidity, OPF was added to a PBS solution whose pH was adjusted to 11.5 with sodium hydroxide. The pH 11.5 was established to be basic enough to buffer the acidity of the OPF, resulting in biocompatible pH of 7.4.

3.5 Cross-linking Time of LAP Photo-Initiator

UV exposure during photo cross-linking the hydrogels was investigated. LAP was investigated as a photo-initiator that would decrease that amount of time VICs were exposed to UV light. All the OPF/PEG hydrogels cross-linked with different concentrations of LAP (0.03, 0.05, 0.075 wt%) and varying UV intensities (2, 5, 10 mW/cm²) for 3 time periods (1.0, 1.5, 3.0 minutes) and had a swelling ratio roughly 7.0 (Figure 4-8). There were not any statistical differences between the swelling ratio when comparing LAP concentration, UV intensities, or time periods. The sol fraction of all the groups were between 0.4-0.5. Although there was not any statistical difference found when comparing LAP concentration, UV intensities, or time periods, there was a general trend that indicated that as the LAP concentration increased the sol fraction decreased.

3.6 Cytotoxicity of Leachable Components & the Hydrogel Solution

The components that VICs encounter: leached products from the OPF/PEG hydrogel, the hydrogel solution (OPF, PEGDA, LAP, PBS), and the individual components OPF and
PEGDA of the hydrogel solution, were tested for their potential cytotoxicity. VICs were exposed to treatments for 20-minutes, indicative of the time needed to process and encapsulate the cells. The conditioned media was created by soaking a cross-linked OPF/PEG hydrogel (1mm:6mm H:D) in 2mL of media for 24 hours. After the hydrogel had been removed, the conditioned media was applied to the cells for 20 minutes. Images of VICs treated with the conditioned media show that there was high cell survival and cells appeared to stay attached to the TCPS well surface (Figure 4-9). Additionally, VICs presented a spindle morphology similar to the live control, indicating that leached products in the conditioned media had little effect on VIC viability and morphology.

Results from the hydrogel solution treatment indicated that there is cell death occurs when VICs are exposed to the hydrogel solution used to suspend cells prior to encapsulation. This is evident by the number of cells that are dead and the morphology of the VICs found in the fluorescent images (Figure 4-9). Valvular interstitial cells presented a rounded morphology, contradictive to the spindle shape found in the live control. Moreover, the VIC cells’ membrane appeared to be forming and releasing vesicles.

Based on results from the hydrogel solution treatment, the components OPF and PEGDA of the hydrogel solution were further investigated for their cytotoxicity. A solution of OPF (20.83 wt%) was made in PBS, with a final pH 7.4. Results from the OPF solution treatment showed high cell viability, with VICs adhered to the plate surface. Furthermore, VICs presented a spindle morphology (Figure 4-9). The PEGDA (Mₙ 575) linker was dissolved in regular PBS (pH 7.4) at a concentration of 4.17 wt%. VICs responded to the PEGDA solution treatment like they did with the hydrogel solution. There was significant amount of cell death and lift off the plate surface (Figure 4-9). Additionally, the cells’
membrane also showed vesicle formation, consistent with the hydrogel solution findings (Figure 4-10).

Overall, the OPF solution and conditioned media showed high cell viability with VICs being in an activated morphology. The hydrogel solution which the VICs come into direct contact during processing and encapsulation is causing significant cellular stress. Specifically, the PEGDA (Mn 575) was shown to be cytotoxic.

4. Discussion

Through visual observation, OPF had material properties (white powder) similar to its PEG (Mn 1,000) precursor. Moreover, due to the polymer having a majority of PEG, OPF readily dissolved into the PBS solution, which is advantageous for encapsulating cells. The cell attachment biomolecule, PEG-RGD, was synthesized through the replacement of a succinimide group in the off the shelf acrylate-PEG-succinimide molecule with CGRGDS peptide sequence.34,45-47 Chemical analysis through 1H-NMR showed that the peptide was attached to the acrylate-PEG molecule and succinimide was removed through the presence of the acrylate and the PEG and the absence of the succinimide peaks (Figure 4-5). Although peptide peaks were hard to delineate due to the overlap with PEG peaks, the NMR spectrum was comparable to that of Peter and Tayalia.48 Upon visual inspection of the final PEG-RGD biomolecule, it was found that the polymer was a white powder, consistent with the acrylate-PEG-succinimide, rather than the airy and less dense consistency of the CGRGDS peptide.

Using the OPF/PEG hydrogel recipe (Table 4-1) a sol-swell analysis was run to determine the largest concentration of MAETAC that could be incorporated into the polymer network. It was expected that as the MAETAC concentration increased to the point that all the excess OPF double bonds were used, MAETAC would start using OPF and PEGDA
double bonds necessary for forming the polymer network leading to an increase in the swelling ratio and, ultimately, loss of hydrogel integrity. A general trend from the sol-swell analysis showed that as the MAETAC concentration increased so did the swelling ratio, consistent with what was expected (Figure 4-6). Additionally, as MAETAC concentration increased over 200 mM it was visually observed that hydrogels were very swollen and susceptible to breaking apart easily. These results from the swelling ratio were comparable to other groups who used OPF or PEGDA based hydrogels for cross-linking MAETAC at concentrations ranging from 50 mM to 400 mM.\textsuperscript{9,10,17–19} Additionally, other groups using OPF and MAETAC to make positively charged hydrogels had similar sol fractions as the OPF/PEG with MAETAC are comparable.\textsuperscript{9,10,12} The sol fraction analysis supported the swelling ratio results. The general trend showed that as MAETAC concentration increased the sol fraction also increased (Figure 4-6) signifying that as the MAETAC concentration increased more of the polymer molecules are failing to cross-link into the polymer network. This supports the idea that as MAETAC increases it starts binding to necessary double bonds for hydrogel formation.

As previously stated, VICs seeded on positively charged surfaces were shown to become osteoblastic-like as indicated by the formation of calcium nodules.\textsuperscript{1} To test whether this same phenomenon occurs in 3D, VICs were to be encapsulated in the positively charged hydrogels with varying concentrations MAETAC and cultured over a period of 7-days. Unfortunately, initial observations of VICs encapsulated in hydrogels made of just OPF/PEG, without any MAETAC, displayed high VIC mortality (Figure 4-7, A). These results were surprising because OPF synthesized with 1 KDa PEG has been shown to have moderate biocompatibility when the OPF macromer was exposed to cells at varying
concentrations (0.4% - 40% w/v) for 2-hours and 24-hours. In addition, PEGDA and OPF has been used extensively for cell encapsulation applications. Therefore, further encapsulation experiments investigated if increasing the concentration of the PEG-RGD biomolecule would increase VIC viability within the control, OPF/PEG hydrogels.

Two concentrations of the cell attachment biomolecule PEG-RGD (1 mM and 2 mM) was tested to determine if the cell death was due to VICs inability to adhere to the polymer scaffold. Results show that both 1 mM and 2 mM concentrations did not support VIC viability (Figure 4-7, C-F) despite that PEGDA based hydrogels mixed with these concentrations have supported cells even VICs previously. It was concluded that VICs were not dying from lack of adherence peptides within the polymer scaffold, but dying before attaching to the RGD groups. Since the OPF/PEG hydrogels with 1 mM and 2 mM PEG-RGD resulted in complete cell death, hydrogels with MAETAC were not tested for VIC viability during encapsulation.

Alternatively, further experiments explored what might have been contributing to hostile conditions leading to cytotoxicity. Specifically, during the synthesis of OPF, HCl is produced as a byproduct. Any residual HCl left in the OPF product could be causing an acid environment, unfavorable for VIC survival when exposed to the hydrogel solution. An analysis of the OPF’s acidity was run. Additionally, the photo-initiator I2959 initially used to form the OPF/PEG hydrogel exposed VICs to UV light for 15-minutes which is a potential cause of cell death. A faster photo-initiator was tested for cross-linking OPF/PEG hydrogels to reduce the time VICs are exposed to UV light. Finally, cytotoxicity of the OPF/PEG hydrogel solution, its components, and uncross-linked material leaching out of the hydrogel was tested on VICs.
To investigate what could be the cause of VIC mortality during encapsulation, it was identified that OPF could still contain residual HCl. The method for synthesizing OPF utilizes nitrogen sparging to mobilize and remove HCl into a neutralizing base bath but there could still be residual HCl remaining in the polymer. When OPF was dissolved in PBS (pH 7.4) at the concentration 20.83 wt% The pH of the PBS solution dropped 4 orders of magnitude. A solution of PBS with a pH of 11.5 was made by adding NaOH and was shown to counteract the acidic polymer resulting with the final hydrogel solution having a pH 7.4. Although the pH was reset to a range that favored cell viability, this did not improve VIC viability when exposed to the hydrogel solution for encapsulation.

In the initial OPF/PEG encapsulation of VICs experiments, the biocompatible photo-initiator I2959 was used. With I2959, to fully encapsulate the VICs in the OPF/PEG hydrogels it required a cross-linking time of 15 minutes (UV intensity 10 mW/cm², 365 nm). It was believed that the UV exposure time of 15-minutes could be a potential cause of cell death. Therefore, the photo-initiator LAP was investigated as an initiator that would speed up the gelation process during encapsulation. Three factors, LAP concentration, UV intensity, and cross-linking time were investigated to help improve cross-linking of the OPF/PEG hydrogels during encapsulation (Table 4-2). Based on the sol-swell analysis, it was found that there was no statistical difference between the UV intensity, LAP concentration, and cross-linking time that improved cross-linking of the OPF/PEG hydrogels (Figure 4-8). Importantly, LAP reduces the time VICs are exposed to UV light by 5x when compared to the I2959 photo initiator.

Results of the sol-swell analysis showed that OPF/PEG hydrogels cross-linked with LAP had similar swelling ratios and improved sol fractions when compared to the OPF/PEG
hydrogels cross-linked with I2959 (Figure 4-8). The improved sol fraction indicates that the LAP initiator is polymerizing more of the hydrogel solution into a polymer network. Unfortunately, the 0.05 wt% of LAP cross-linked with 5 mW/cm² treatment was not handled properly and was excluded from the study. Based on the results, OPF/PEG hydrogels can be formed with the highest cross-linking efficiency by using the LAP concentration.\textsuperscript{54} Furthermore, there is a clear reduction in cross-linking times of LAP for all concentrations and intensities when compared to I2959 (0.05 wt%, 10 mW/cm²). Using LAP should improve the VIC viability during encapsulation because of the reduced exposure to UV light.

Finally, components of the hydrogel solution were tested for their cytotoxicity. Specifically, a conditioned media was made with leached products of the OPF/PEG hydrogels, the hydrogel solution (OPF/PEG/LAP/PBS), a solution of OPF (20.83 wt%) in PBS, and a solution of PEGDA (Mₙ 575) in PBS were administered to the VICs for 20-minutes, replicating the encapsulation processing time. Results indicated that the conditioned media and OPF solutions supported VIC culture.\textsuperscript{44,56}

The 20-minute exposure to the hydrogel and PEGDA solutions were found to be more cytotoxic and induced VIC detachment from the well plate (Figure 4-9). Also, the VICs morphology appeared to be rounded and had lost cytoplasmic volume while producing vesicles. Finally, unknown microspheres were seen attached to VIC membranes (Figure 4-10). It is hypothesized that these microspheres are PEGDA that are attaching to the cells, but future work is needed to confirm this. It is determined that PEGDA (Mₙ 575) was one of the causes of VIC cytotoxicity in the hydrogel solution and therefore a reason cells were not surviving the encapsulation process.
Results concluding that PEGDA ($M_n$ 575) is cytotoxic to VICs are contradictory to some other hydrogel platforms, though prior studies are mixed in this regard. Works that were used to construct the positively charged OPF/PEG showed that OPF hydrogels using PEGDA ($M_n$ 575) as a cross-linker support cell encapsulation and growth.\textsuperscript{33,34,49,57} Additionally, PEGDA $M_n$ 575, 700, 3400 hydrogels used to encapsulate cells were shown to support cell encapsulation but the higher molecular weight PEGDA had increased cell viability during the encapsulation process and through long term cell culture (14 days).\textsuperscript{58} However, other results have shown that PEGDA ($M_n$ 575) at concentrations ranging from 0.1%-10% w/v are cytotoxic to cells when they are exposed to PEGDA for 2-hours and 24-hours. Again, results showed that increasing the molecular weight and decreasing the concentration of PEGDA improved cell viability.\textsuperscript{44,56} Based on current results, it appears that PEGDA ($M_n$ 575) has the latter effect of being cytotoxic to VICs in this OPF/PEG hydrogel platform. Future iterations to improve VIC viability in OPF/PEG should consider increasing the PEGDA cross-linker’s molecular weight.

5. Conclusion

Unlike other OPF based hydrogel systems that have been shown to support 3D culture, the OPF used in this hydrogel platform was synthesized through a newly developed acid scavenger-free method and did not support VIC culture.\textsuperscript{32,36,44,56,59} A few factors related to cell mortality were explored, namely OPF pH, UV exposure time, and PEGDA ($M_n$ 575). It was found that the OPF still had considerable amounts of HCl. Therefore, additional post-processing methods should explore removal of residual HCl from the polymer, rather than offsetting the PBS pH to be basic. Generally, this will reduce the need to match the correct basic pH of the PBS to counter the variation in acidity of the OPF per batch. To reduce the
amount of UV exposure VICs are subjected during cross-linking, the water-soluble photo-initiator LAP has replaced the less water soluble I2959 photo-initiator. Finally, using a PEGDA linker molecule that has a higher molecular than 575 is likely to increase the viability of cells.\textsuperscript{44} Thus, further research to optimize the positively charged hydrogel system should consider using higher molecular weight PEGDA, such as PEGDA $M_n$ 3400.
Figures

**Figure 4-1.** Comparison of the aortic valve with (A) healthy leaflet tissue and (B) stenotic leaflet tissue.
Figure 4-2. Valvular interstitial cell phenotype *in vitro* conditions. The morphological characteristics of quiescent, activated, and osteoblastic VICs. Differentiation potential of VICs for healthy and pathological states.
**Figure 4-3.** The polymer network formed from photo cross-linking OPF and PEGDA with the charged monomer MAETAC and PEG-RGD biomolecules for encapsulating VICs. Two photo-initiators (I2959 or LAP) were explored as for creating positively charged hydrogels.
Figure 4-4. $^1$H-NMR spectrum of OPF. Solvent was deuterated chloroform. Peaks (a,b) indicate the olefin group of the fumarate monomer, (d,e) indicate the PEG ($M_n$ 1000), (c) indicates the ester bond formed when the PEG and fumarate react together.
Figure 4-5. $^1$H-NMR of synthesized PEG-RGD biomolecule used for cell attachment. (TOP) is the acrylate-PEG-succinimide ($M_n$ 3400) polymer being functionalized with the CGRGDS peptide sequence. (BOTTOM) show the Acr-PEG-RGD biomolecule synthesized.
Figure 4-6. Swelling ratio (A) and sol fraction (B) analysis of positively charged hydrogels. Increasing molar concentrations of MAETAC were photo cross-linked into OPF/PEG hydrogels and compared to an OPF/PEG control (n = 6). *Significantly different from each other, (A) p=0.0433 and (B) p=0.00927 (400 mM to 50 mM), p=7.04x10^{-5} (400 mM to OPF/PEG)
Figure 4-7. Brightfield and fluorescent images of VICs encapsulated in OPF/PEG hydrogels with different concentrations of PEG-RGD biomolecule right after encapsulation. (A, B) shows VICs encapsulated in hydrogels without any biomolecules. (C, D) are VICs encapsulated in hydrogels with 1 mM of the PEG-RGD, and (E, F) indicated VICs in hydrogels with 2 mM concentration of PEG-RGD. (Live = Green, Dead = Red)
Figure 4-8. Sol-swell analysis of OPF/PEG hydrogels photo cross-linked with varying concentrations of LAP (0.03, 0.05, 0.075 wt%) and varying UV concentrations (2, 5, 10 mW/cm$^2$). OPF/PEG (OPF 20.87 wt%, PEGDA 4.17 wt%) hydrogels in PBS (pH 7.4), without PEG-RGD biomolecules, were photo cross-linked with LAP. No significant differences were found between UV intensities, LAP concentrations, or cross-linking time. (n = 4)
Figure 4-9. Cytotoxicity of analysis of the conditioned media with leached OPF/PEG hydrogel components, the uncross-linked hydrogel solution VICs are suspended in for encapsulation, and the hydrogel components OPF (20.83 wt%) and PEGDA (4.17 wt%) in PBS (pH 7.4). Exposure time to each treatment was 20-minutes to replicate the time VICs are exposed to the uncross-linked components before encapsulation and the initial cross-linked OPF/PEG hydrogel. Live = Green, Dead = Red, Scale = 100 µm
Figure 4-10. Brightfield images of VICs exposed to (A) PEGDA 4.17 wt% and (B) OPF/PEG hydrogel solution for 20-minutes. Comparison to a (C) live control that had been exposed media, and a (D) dead control that was exposed to 70% ethanol for 30 minutes. White arrows indicated vesicle formation within and outside the VICs. Yellow arrows indicate residual PEGDA spheres.
Tables

Table 4-1. The amounts of each component of the positively charged hydrogel recipe. Indication of the different concentrations of MAETAC, and the conversion of the MAETAC molar concentration to a weight %.

<table>
<thead>
<tr>
<th>MAETAC Concentration (mM)</th>
<th>MAETAC (wt%)</th>
<th>OPF (wt%)</th>
<th>PEGDA (wt%)</th>
<th>Irgacure (Wt%)</th>
<th>PBS (Wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>20.83</td>
<td>4.17</td>
<td>0.05</td>
<td>74.95</td>
</tr>
<tr>
<td>50</td>
<td>1.04</td>
<td>20.83</td>
<td>4.17</td>
<td>0.05</td>
<td>73.91</td>
</tr>
<tr>
<td>100</td>
<td>2.08</td>
<td>20.83</td>
<td>4.17</td>
<td>0.05</td>
<td>72.87</td>
</tr>
<tr>
<td>200</td>
<td>4.15</td>
<td>20.83</td>
<td>4.17</td>
<td>0.05</td>
<td>70.8</td>
</tr>
<tr>
<td>400</td>
<td>8.31</td>
<td>20.83</td>
<td>4.17</td>
<td>0.05</td>
<td>66.64</td>
</tr>
</tbody>
</table>
**Table 4-2.** Cross-linking kinetic study parameters measured through sol-swell of the final hydrogels. LAP concentration, UV Intensity, and Cross-linking time was test on the OPF/PEG hydrogels. (n = 4)

<table>
<thead>
<tr>
<th>LAP Concentration (wt%)</th>
<th>UV Intensity (mW/cm²)</th>
<th>Cross-Linking Time (Minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td>0.05</td>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td>0.075</td>
<td>10</td>
<td>3.0</td>
</tr>
</tbody>
</table>
References


(11) De Luca, I.; Di Salle, A.; Alessio, N.; Margarucci, S.; Simeoni, M.; Galderisi, U.; Calarco, A.; Peluso, G. Positively Charged Polymers Modulate the Fate of Human


(35) Daly, W. T.; Knight, A. M.; Wang, H.; de Boer, R.; Giusti, G.; Dadsetan, M.; Spinner, R. J.; Yaszemski, M. J.; Windebank, A. J. Comparison and Characterization of Multiple


(59) Coombs, K. Designing Synthetic Environments to Control Valvular Interstitial Cells In Vitro, University of New Mexico, Health Sciences Center, 2018.
Chapter 5

Poly(1,2-Butylene Fumarate) and Poly(1,3-Butylene Fumarate)

Characterization for Use in Tissue Engineering

Christian T. Denny¹,², Jasmine Jackson³, Charles Easterling, PhD⁴, Elizabeth L. Hedberg-Dirk, PhD¹³, Christina Salas, PhD¹⁵,⁶

¹Center for Biomedical Engineering, University of New Mexico, Albuquerque, New Mexico, USA.

²Biomedical Engineering Graduate Program, University of New Mexico, Albuquerque, New Mexico, USA.

³Department of Chemical and Biological Engineering, University of New Mexico, Albuquerque, New Mexico, USA.

⁴Center for Integrated Nanotechnologies, Sandia National Laboratories/Los Alamos National Laboratory, Albuquerque, New Mexico, USA.

⁵Department of Orthopaedics and Rehabilitation, The University of New Mexico Health Sciences Center, Albuquerque, New Mexico

⁶Department of Mechanical Engineering, The University of New Mexico, Albuquerque, New Mexico
Abstract

Current methods to treat ligament tears range from primary ligament repair to full reconstruction of the damaged area but fail to replicate the biomechanical and biochemical gradients found within the bone-ligament (BL) interface. Advances in biomaterials and biomolecule research have created mechanical and biochemical gradients like the bone phase but research into biomaterials that replicate the whole BL enthesis is needed. Poly(butylene fumarate) is linear polyester that has unsaturated double bonds available for crosslinking. This material can be systematically engineered to have varying material properties through the selection of the butylene monomer. This work synthesizes poly(1,2-butylene fumarate) (1,2-PBF) and poly(1,3-butylene fumarate) (1,3-PBF) and developed them into photo crosslinkable solutions and cast them into films. Through nuclear magnetic resonance (NMR) and Fourier-transform infrared spectroscopy (FTIR) both PBF polymers were shown to be successfully synthesized. Gel permeation chromatography revealed that 1,2-PBF had a number-average molecular weight (M_n) of 1,380 ± 127 g/mole, weight-average molecular weight (M_w) of 2,139 ± 199 g/mole, polydispersity index (PDI) of 1.55 ± 0.06, and a glass transition temperature (T_g) of -20.0°C. Correspondingly, 1,3-PBF had a M_n of 3,315 ± 116 g/mole, M_w of 6,618 ± 139 g/mole, PDI of 2.00 ± 0.04, and a T_g of -27.5°C. The FTIR spectrum of both PBF films showed a high degree of crosslinking. It was also found that 1,3-PBF was stiffer than 1,2-PBF. Further research will aim to 3D bioprint the materials into porous scaffolds and test biocompatibility.
1. Introduction

In the United States, sports and recreational-related sprains and strains account for 4,262,000 injuries annually.\(^1\) Sprains and strains stretch and, potentially, tear tendons and ligaments causing instability, pain, and loss of mobility in joints. Current surgical approaches aim to restore the biomechanical function of the torn ligament through primary ligament repair and pinning or ligament reconstruction with screw fixation, tendon, or bone-ligament-bone (BLB) grafts.\(^2\)–\(^6\) Commonly used reconstruction material options are typically classified as autograph or non-autograph. The autographic material is beneficial because it matches physiological structure and mechanical strength but can lead to donor-site morbidity and limited donor tissue.\(^7\) Non-autograph options reduce the chance of donor-site morbidity but lack the mechanical and biochemical cues needed to restore bone and ligament. Generally, failures of reconstructive materials occur at the bone-ligament (BL) interface. This is due to the high-stress concentration at the interface and inadequate regeneration of the enthesis.\(^8\)–\(^15\) Due to these problems, there is a need for treatment options that aim to restore the biomechanical and biochemical function of the BL interface while simultaneously allowing tissue regeneration.\(^16\)–\(^19\)

In an attempt to reduce BL reconstructive failures, BLB constructs are whole ligament implants that are either taken from donor tissue or fabricated \textit{ex situ} and focus on long-term regeneration of the BL interface.\(^20\)–\(^22\) Precisely, BLB constructs aim to recapitulate the biomechanical and biochemical gradients found in the BL \textit{milieu} to restore natural function to the joint. Replicating the interface encompasses a complex and dynamic mechanical gradient starting at the highly compressive strong bone phase and transitioning to the highly elastic ligament phase.\(^15,23,24\) A similar complex biochemical gradient can be found
too. Within the interface, the BL enthesis is a short transitional phase that adjoins the bone and ligament phases. The enthesis starts with long type I collagen fibers inserted into uncalcified-fibrocartilage, then progresses into mineralized-fibrocartilage, and finally ends in bone material.\textsuperscript{18,25} Current natural BLB implants maintain these biomechanical and biochemical gradients but are limited by tissue availability and donor-site morbidity.\textsuperscript{20,21} Some synthetically fabricated BLB alternatives have been developed but do not accurately replicate the natural BL environment. Therefore, there has been a considerable amount of research in further developing synthetic BLB devices.

There has been the successful development of scaffolds with synthetic polymers that have similar mechanical gradients as the bone and ligament environment and have been shown to support cell growth and differentiation of mesenchymal stem cells but there is a need for biomaterials that replicate the mechanical properties of the BL enthesis.\textsuperscript{23,24} Additionally, the incorporation of bioactive molecules into phases of the synthetic BLB has been used to make biochemical gradients that compartmentally differentiate cells types in their bone, fibrocartilage, and ligament phenotypes which are biocompatible and functional \textit{in vivo}.\textsuperscript{16–18,24,26,27} These results demonstrate the need to combine the biomaterial and bioactive components into architecturally complex BLB devices that accurately replicate the biomechanical, biochemical, and material organization found in the bone, fibrocartilage, and ligament for regeneration of the BL interface.

Advances in additive manufacturing have made it possible to mimic the material architecture while maintaining the biomechanical and biochemical gradients found in the BL interface. Three-dimensional (3D) bioprinting is a commonly used manufacturing technique that can use a variety of biomaterials to create porous bone constructs with varying degrees
of mechanical strength and osteoinductive properties. Electrospinning is another additive manufacturing technique that can make nanoscale fibers out of biomaterials in a variety of patterns. A specific type of electrospinning, near-field electrospinning, makes nanoscale fibers but has the added advantage of controllable fiber deposition. This allows for systematic fiber deposition into geometries that are optimal for ligament tensile strength and fibroblast growth. A new hybrid bioprinting system has been developed that combines 3D bioprinting and near-field electrospinning. This hybrid printer has the benefit of printing and spinning biomaterials to create the BLB composite construct with a porous bone phase and filamentous ligament phase. This technique is promising for making highly detailed constructs with architecture that replicates the material organization of the BL interface. While this 3D printing can make high fidelity constructs, new biomaterials and bioink solutions are needed to replicate the BL interface more accurately, specifically the enthesis, and stimulate tissue regeneration.

Of interest are materials that are designed specifically for bone and mineralized fibrocartilage. Some biomaterials that have been shown to grow bone tissue consist of ceramics (hydroxyapatite, calcium phosphate, and bioactive glass) and synthetic polymers (polycaprolactone, poly(lactic-co-glycolic acid), polymethyl-methacrylate, poly(propylene fumarate). Recently Lui et al. have 3D printed a multiphasic BLB scaffold out of medical-grade polycaprolactone and showed that the scaffold demonstrated compartmentalized tissue regeneration of the bone and ligament region, while maintaining structural integrating in vivo. A hybrid bone tissue scaffold was fabricated using 3D printing and electrospinning of poly(ethylene oxide terephthalate)-poly(butylene terephthalate) and coated with calcium phosphate. These scaffolds were found to support
stem cell attachment, proliferation, and differentiation into osteogenic cells.\textsuperscript{27} Furthermore, polycaprolactone, hydroxyapatite, and β-tricalcium phosphate were combined and 3D printed into porous scaffolds that had mechanical and biochemical gradients. Through these gradients, it was shown that mesenchymal stem cells could attach and differentiate into osteoblasts.\textsuperscript{23,24}

Poly(propylene fumarate) (PPF) is a synthetic fumarate-based polymer that has been used extensively in bone tissue engineering. The linear polyester is favored for its multiple unsaturated double bonds that can be used for photo or thermal crosslinking and its ability to biodegrade through hydrolysis.\textsuperscript{32,34–39} Likewise, PPF has been shown to have optimal mechanical properties for 3D bioprinting stiff porous constructs that have similar mechanical strength as bone tissue.\textsuperscript{23,29,32} The mechanical stiffness and surface chemistry have also been shown to support osteoblast attachment and growth over extended culture periods.\textsuperscript{28,34} When combined with bioactive ceramics and growth factors, PPF has been considered a suitable material for building BLB scaffolds.\textsuperscript{32,40} Unfortunately, PPF has an extended degradation time which is not conducive to scaffold remodeling and bone regeneration.\textsuperscript{41} There is a need for tailored materials that have similar properties as PPF and improve tissue regeneration.\textsuperscript{23,24,33}

Poly(butylene fumarate) (PBF) is a similar fumarate-based polymer that has potential as a 3D printable bone material. Like PPF, PBF contains unsaturated double bonds and is hydrolytically biodegraded, but unlike PPF, it can be chemically engineered to have different material properties through its butylene group. It is believed the additional carbon in the butane and the presence of a side group, like the ethyl group in 1,2-butane, will alter the material properties of the polymer. Creating a looser polymer network compared to PPF, and
when crosslinked, causes altered mechanical and material properties. Notably, a looser network allows for increasing degradation to a rate that is favorable for bone regeneration.\textsuperscript{41,42} Poly(butylene fumarate) has been previously synthesized with different conformations of butane (1,3-butane, 1,4-butane) and in co-polymers for tissue engineering purposes.\textsuperscript{41–45} Further investigation into PBF with different butylene conformations is of interest for developing additional biomaterials for tissue engineering the BL milieu.

We recently synthesized poly(1,2-butylene fumarate) (1,2-PBF) and poly(1,3-butylene fumarate) (1,3-PBF) and are interested in characterizing the polymers for use in bone tissue engineering (Schematic 5-1). To date, poly(1,2-butylene fumarate) (1,2-PBF) has not been published as a biomaterial for any tissue engineering applications and is being investigated as a new biomaterial. In this work both polymers will be synthesized, characterized, and made into photo crosslinkable solutions, and cast into films to study the polymer's ability to form polymer networks.

2. Materials and Methods

2.1 Poly(Butylene Fumarate) Synthesis

Poly(1,2-butylene fumarate) and poly(1,3-butylene fumarate) were synthesized via an acid free polycondensation reaction.\textsuperscript{46,47} Fumaryl chloride (FuCl, TCI, F0152) was distilled before use. Briefly, one mole of 1,2-butanediol (1,2-BD) or 1,3-butanediol (1,3-BD) was dissolved in dichloromethane (DCM, VWR, BDH1113) in a 3-neck round bottom flask. The reaction was maintained under a nitrogen blanket to impede oxygen inhibition of the polymerization. The solution was stirred vigorously while a 1:1 molar ratio of FuCl to butanediol (BD) was added dropwise over 4-6 hours. Throughout the reaction, removal of hydrochloric acid (HCl) byproduct was accomplished by nitrogen gas sparging using a fine
fritted gas dispersion tube (Chemglass, CG-203-01) at a flow rate of 2.0 SCFH. The removed HCl was neutralized in a 1 M NaOH (VWR, 221465) in ethanol (Koptec, V1001). The reaction was allowed to progress for 48-hours under continual sparging with periodic DCM refills to maintain a constant level within the reaction vessel. At the completion of the reactions, DCM was removed via rotary evaporation (Buchi, R-215). The PBF was then resuspended in chloroform and washed with deionized water 3 times. The polymer was dried with a brine wash followed by the addition of magnesium sulfate to the polymer solution to completely remove residual water. The solution was vacuum filtered to remove the magnesium sulfate. The solvent was removed via rotary evaporation and all polymers were stored at -20°C under dark conditions.

2.2 Chemical and Material Properties of Poly(Butylene Fumarate)

Proton and carbon nuclear magnetic resonance (\(^1\)H-NMR, \(^{13}\)C-NMR) imaging was performed to assess the chemical structure of both PBF polymers using the Avance III Solution 300 (300 MHz, Bruker, Massachusetts). Characterization was performed using borosilicate NMR tubes (5 mm diameter, Wilmad, New Jersey) with deuterated chloroform (Millipore Sigma, Germany) as the solvent. The molecular weight of the polymers was analyzed using gel permeation chromatography (GPC) on the Agilent 1260 Infinity II (Santa Clara, CA) with two Agilent PL-gel 5 μm MIXED-C columns running in tetrahydrofuran (THF, 1.0 mL/min at 30°C) with a polystyrene standard (Agilent). Differential scanning calorimetry (DSC) was performed on the STA 449 F1 Jupiter (NETZSCH, Germany) under nitrogen atmosphere to determine glass transition temperature. Samples were cooled at a 5 K/min rate and heated at a rate of 3 K/min, respectively.

2.2 Poly(Butylene Fumarate) Films
To create polymerization solutions, PBF was mixed with a diethyl fumarate solution (DEF, Millipore Sigma, D95654) in a 9:1 weight ratio of PBF to DEF solution. To make the DEF solution, 0.3 wt% of photo-initiator phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide (BAPO, Millipore Sigma, 511447) was dissolved in 9.7 wt% of DEF and minimal (500μL:1g BAPO) chloroform (Millipore Sigma, CX1055).\textsuperscript{30,35} The final PBF:DEF solution was placed in a vacuum oven for 24-hours to remove residual chloroform.

The solutions were then injected between two glass slides held 1 mm apart with a Teflon spacer. The mold was placed in a UV Crosslinker box (Analytik Jena, CL-1000) for 30-minutes (365nm, 2 mW/cm\textsuperscript{2}). Crosslinked films were removed from the molds and used for analyses.

2.3 Characterization of Crosslinked Poly(Butylene Fumarate)

Fourier transform infrared (FTIR) spectra were obtained from 1,2-PBF and 1,3-PBF polymers along with associated crosslinked films on a Bruker IFS 66vS (Bruker Optik, Germany). All samples were analyzed using a grazing angle attenuated total reflectance (GATR) with a fixed 65° incident angle on a hemispherical germanium crystal (Harrick Scientific Product Inc., Pleasantville, NY). 256 scans per sample were collected at a 2 cm\textsuperscript{−1} resolution from 4000 cm\textsuperscript{−1} to 700 cm\textsuperscript{−1} using liquid nitrogen cooled MCT detector.

2.4 Statistics

All measured data were reported as average ± standard deviation. Statistics were performed using Microsoft Excel.

3. Results

3.1 Nuclear Magnetic Resonance Spectroscopy
Poly(1,2-butylene fumarate) and poly(1,3-butylene fumarate) were successfully synthesized using a polycondensation reaction that polymerized 1,2-BD or 1,3-BD with FuCl (Schematic 5-1). The molecular structure of PBFs was confirmed using $^1$H-NMR and $^{13}$C-NMR analyses. Representative peaks for the fumarate group were present at 6.5-7.0 ppm on the $^1$H-NMR spectra (Figure 5-1). The ethylene peaks associated with the 1,2-butylene were located at 0.8-1.1 ppm and 1.4-1.9 ppm. While the 1,3-butylene methyl peaks were located at 1.0-1.4 ppm and 1.6-2.2 ppm.\textsuperscript{1,2} Moreover, it was observed that BD peaks shift downfield after polymerization indicating increased shielding of the butane protons in the PBF polymer.

In the $^{13}$C-NMR spectrum of the 1,2-PBF, three carbon peaks appear for the BD group at 72-76 ppm (CH), 64-67 ppm (CH$_2$-O), 23-25 ppm (CH$_2$), and 8-10 ppm (CH$_3$). Additionally, BD groups in the 1,3-PBF spectrum appear at 68-72 ppm (CH), 60-65 ppm (CH$_2$-O), 33-40 ppm (CH$_2$), and 18-24 ppm (CH$_3$). The fumaric group can be found at peaks 132-136 ppm (C=C) and 163-166 ppm (C=O) for both 1,2- and 1,3-PBF spectra (Figure 5-2).\textsuperscript{38,43,48}

3.2 Fourier-Transform Infrared Spectroscopy

Upon further chemical analysis of the PBF polymers, FTIR also confirmed that 1,2-PBF and 1,3-PBF were successfully synthesized through the presence of characteristic bonds. The unsaturated double bond peaks at 979 cm$^{-1}$ (C-H, Bending), 1644 cm$^{-1}$ (C=C, Stretching), and 2973 (C-H, Stretching) indicated the presence of the fumarate group (Figure 5-3).\textsuperscript{43,49} Peaks at 1157 cm$^{-1}$ (C-O, Stretching) and 1297 cm$^{-1}$ (C-C, Stretching) also indicated the presence of the butane group (Table 5-2).\textsuperscript{50} Finally, peaks located at 1157 cm$^{-1}$ (C-O, Stretching) and 1725 cm$^{-1}$ (C=O, Stretching) confirm that esterification of butane and fumarate has occurred.
3.3 Molecular Weight and Glass Transition Temperature

Molecular weight analysis showed that 1,2-PBF has a number-average molecular weight (\(M_n\)) of 1,380 ± 127 g/mole, weight-average molecular weight (\(M_w\)) of 2,139 ± 199 g/mole, and a polydispersity index (PDI) of 1.55 ± 0.06 (Table 5-1).\(^{34,35}\) While 1,3-PBF had a \(M_n\) of 3,315 ± 116 g/mole, \(M_w\) of 6,618 ± 139 g/mole, and a PDI of 2.00 ± 0.04. Based on the \(M_w\) of 1,2-PBF and 1,3-PBF the double bonds per molecule are 11-14 and 38-40, respectively. Additionally, the glass transition temperatures were found to be -20.0°C for 1,2-PBF and -27.5°C for 1,3-PBF (Table 5-1).\(^{43,44,49}\)

3.4 Characterization of Poly(Butylene Fumarate) Films

Poly(butylene fumarate) was combined with diethyl fumarate and BAPO and crosslinked through UV initiation into hard plastic films.\(^{30,35}\) Comparison of FTIR spectrums of 1,2-PBF and 1,3-PBF polymers to their corresponding films was used to evaluate the degree of crosslinking. Specifically, the absence of the fumarate double bond peak at 1644 cm\(^{-1}\) (C=C, Stretching) was used to confirm that the unsaturated bonds were indeed used during UV crosslinking to form the polymer network. The 1,2-PBF polymer has a distinct peak at 1644 cm\(^{-1}\) peak but was not present in the crosslinked 1,2-PBF film (Figure 5-4). Correspondingly, the 1,3-PBF and the crosslinked 1,3-PBF film showed a similar result, indicating most of the fumarate doubles bonds were used during the UV crosslinking process. There are differences in material properties when handled. The 1,2-PBF was found to be more flexible and 1,3-PBF scaffolds were stiffer.

4. Discussion

The bone-ligament interface is a complex structure made up of hard and soft materials that adapt to high compressive tensile loads to maintain stability. It consists of a
complex biomechanical and biochemical gradient. Tissue engineering has aimed to mimic these mechanical and chemical gradients to develop a device that can be used for studying and treating damaged ligaments.\textsuperscript{26} To accomplish making a complex interface, such as the bone-ligament, new biomaterials and methods are needed. As such, this work has investigated PBF’s potential as a biomaterial for use as a bioink to 3D print the bone phase. Specifically, two variations of PBF were synthesized using 1,2-BD and 1,3-BD (\textbf{Schematic 5-1}).\textsuperscript{43,44,46} Through $^1$H-NMR and $^{13}$C-NMR analyses it was confirmed that butanediol successfully reacted with FuCl (\textbf{Figures 5-1 and 5-2}).\textsuperscript{38,43,44,48} Additionally, there were residual peaks associated with butanediol suggesting that there was butanediol left in the final product of both PBF polymers. Further post-processing steps, like size-exclusion chromatography, should be tested for purifying the final PBF product. In a $^1$H-NMR comparison of the corresponding butanediol to PBF, it was found that butane-associated peaks, like the methyl and ethyl groups, shift downfield after polymerization. This can be explained by butane protons being more shielded by the fumarate groups.

Additionally, $^1$H-NMR peak comparison of 1,2-PBF showed that the olefin peak (6.5-70 ppm) was half of the ethyl peaks (0.8-1.1 ppm) (\textbf{Figure 5-1}). Theoretically, a 1:1 molar ratio of BD:FuCl should produce the comparable peak intensities of the olefin to ethyl peaks. It is hypothesized that the discrepancy in the olefin peak intensity is again contributed to reduced polymerization during the synthesis. The residual unreacted FuCl is removed from the final 1,2-PBF product during the washing steps. When FuCl is neutralized and washed out of the polymer. Conversely, 1,2-BD is miscible in water and is retained in the polymer solution throughout the post-processing steps. The presence of butanediol in the final PBF polymer would show up as additional peaks in the NMR spectrum, which can be seen in the
$^1$H-NMR and $^{13}$C-NMR spectra (Figures 5-1 and 5-2). Further purification steps should be investigated to completely remove unreacted butanediol from the final 1,2-PBF polymer.

FTIR analyses also confirmed the polymerization of butanediol with FuCl through the presence of the ester bond stretching peaks (Figures 5-3 and Table 5-2). Although 1,2-PBF and 1,3-PBF spectra showed characteristic PBF peaks, they lacked a broad hydroxyl peak found past 3000 cm$^{-1}$. The absences of these peaks suggest that a large fraction of PBF molecules do not contain hydroxyl end groups, but rather carboxyl end groups. A 1:1 molar ratio of BD:FuCl theoretical would have a mixture of hydroxyl and carboxyl terminated polymers in one sample. Ideally, the hydroxyl-terminated end groups are preferred due to the acidity of the carbonyl. Altering the BD:FuCl molar ratio to have more butanediol would increase the chances of more hydroxyl-terminated PBF molecules.

The material properties associated with polymers are correlated to their molecular weight. To investigate 1,2-PBF and 1,3-PBF as potential biomaterials for 3D printing applications their molecular weights were analyzed. The 1,2-PBF had a $M_w$ of 2,139 ± 199 g/mole, which is ~3 times smaller than the molecular weight of 1,3-PBF 6,618 ± 139 g/mole for the same reaction time and conditions (Table 5-1). The difference in molecular weight could be contributed to steric hindrance during synthesis, wherein 1,2-BD’s hydroxyl groups were more sterically hindered, thereby reducing the chances of polymerization with FuCl. The resulting difference in molecular weight affected the viscosity and $T_g$ of the PBF polymers. It was visually observed that 1,3-PBF had a higher viscosity than the 1,2-PBF at room temperature, and 1,2-PBF had a higher $T_g$ -20°C than the 1,3-PBF -27°C (Table 5-1). It was found that the crosslinked films of PBF had different stiffnesses. Upon visual handling, the 1,2-PBF films were more flexible than the 1,3-PBF scaffolds. The differences
in material properties are due to molecular weight and the BD structure in the polymer backbone. Particularly, 1,2-PBF contains 11-14 double bonds per molecule compared to 38-40 double bonds that 1,3-PBF contains. The amount of double bonds per molecule, in turn, affects the organization of the polymer network during UV crosslinking. Moreover, the butane side group also contributes to the polymer packing. The 1,2-BD group has an ethyl side group that reduces polymer chain packing and the methyl group in 1,3-PBF allows for a tighter polymer network causing the bulk properties to be stronger.

Photo initiation is a popular method for starting free radical polymerization during 3D printing. Therefore 1,2-PBF and 1,3-PBF were made into UV crosslinkable solutions using phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide. It was shown that both 1,2-PBF and 1,3-PBF solutions were cast into films that fully crosslinked after 30-minutes of exposure to 2 mW/cm² UV light (365 nm). To analyze the degree of crosslinking of the films, FTIR spectrums were taken, and the unsaturated double bond peaks of the polymer and crosslink films were compared. A distinct peak at 1644 cm⁻¹ that is assigned to the fumarate double bond can be observed in 1,2-PBF and 1,3-PBF polymer FTIR spectrums, but, after crosslinking, the film spectrums show a significant reduction in intensity, suggesting a high degree of crosslinking is occurring (Figure 5-4).

5. Conclusions

Through chemical and material analysis it was shown that 1,2-PBF and 1,3-PBF were successfully synthesized into a linear polyester polymer with multiple functional double bonds. It was found that 1,2-PBF differed from 1,3-PBF in molecular weight and glass transition temperature. Polymers were UV crosslinked into films. The differences in molecular weight and chemical structure of butane in 1,2-PBF and 1,3-PBF polymers
translated into differing material properties, where 1,2-PBF films were found to be more flexible than the 1,3-PBF scaffolds. Results show that 1,2-PBF, and 1,3-PBF are promising biomaterials for future bone tissue engineering and 3D bioprinting applications. Further investigation into the printability of the PBF solution is necessary for future use in 3D printing the bone phase of the bone-ligament device.
Schematic 5-1. Set-up for synthesizing PBF using an acid free, polycondensation reaction (A). Chemical reaction for making both types of PBF (1,2-PBF and 1,3-PBF) from FuCl and 1,2-BD or 1,3-BD, utilizing nitrogen sparging (B). Adapted from (46)
Figure 5-1. Proton NMR of 1,2-PBF (Top) and 1,3-PBF (Bottom). In-laid spectrum are proton NMR of corresponding butanediol only.
Figure 5-2. Carbon NMR of 1,2-PBF (Top) and 1,3-PBF (Bottom). Both types of PBF were run in CDCl$_3$ solvent (δ 77.0, triplet).
Figure 5-3. Fourier-Transform infrared spectrum of 1,2-PBF and 1,3-PBF polymer and the corresponding 1,2-PBF and 1,3-PBF scaffolds.
Figure 5-4. Fumarate peak 1644 cm\(^{-1}\) (C=C, Stretching) comparison of (A) 1,2-PBF polymer vs crosslinked scaffold and (B) 1,3-PBF polymer vs crosslinked scaffold.
### Tables

**Table 5-1.** The molecular weight of synthesized 1,2-PF and 1,3-PBF with corresponding polydispersity index. (n = 4)

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$T_g$ (°C)</th>
<th>$M_n$ (g/mole)</th>
<th>$M_w$ (g/mole)</th>
<th>PDI</th>
<th>Double Bonds per Molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-PBF</td>
<td>-20.0</td>
<td>1380 ± 127</td>
<td>2139 ± 199</td>
<td>1.55 ± 0.06</td>
<td>7-9 (Mn) 11-14 (Mw)</td>
</tr>
<tr>
<td>1,3-PBF</td>
<td>-27.5</td>
<td>3315 ± 116</td>
<td>6618 ± 139</td>
<td>2.00 ± 0.04</td>
<td>18-20 (Mn) 38-40 (Mw)</td>
</tr>
</tbody>
</table>
**Table 5-2.** FTIR spectral peaks of poly(butylene fumarate) polymers and scaffolds.\(^5^0\)

<table>
<thead>
<tr>
<th>Spectral Response</th>
<th>Peak Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>C—H bending of trans CH=CH for PBF</td>
<td>979 cm(^{-1})</td>
</tr>
<tr>
<td>C—O stretching of ester linkage for BD</td>
<td>1157 cm(^{-1})</td>
</tr>
<tr>
<td>C—C stretching for BD</td>
<td>1297 cm(^{-1})</td>
</tr>
<tr>
<td>—CH(_3) asymmetric bending for BD</td>
<td>1469 cm(^{-1})</td>
</tr>
<tr>
<td>C=C stretching of —CH=CH— for PBF</td>
<td>1688 cm(^{-1})</td>
</tr>
<tr>
<td>C=O stretching of ester for PBF and BD</td>
<td>1725 cm(^{-1})</td>
</tr>
<tr>
<td>C—H</td>
<td>2878 cm(^{-1})</td>
</tr>
<tr>
<td>C=CH(_2)</td>
<td>2973 cm(^{-1})</td>
</tr>
</tbody>
</table>
References


Chapter 6

Conclusions

In the last 20 years, tissue engineering has seen rapid development in new biomaterials, cell types, and signaling molecules through the assimilation of concepts from related fields of study, like materials science, rapid prototyping, nanotechnology, and cell biology. Particularly, in the field of biomaterials, there have been advances in smart biomaterials and additive manufacturing that have given engineers the ability to design nano- and microscale-scale scaffolds to replicate the native tissue organization and respond to their environments. Within the field of biomaterials, fumarate-based polymers have been foundational in the development of bone, cartilage, and soft tissue engineering.

Poly(propylene fumarate) (PPF) and oligo(poly(ethylene glycol) fumarate) (OPF) have been studied extensively for tissue engineering and drug delivery. The linear synthetic polymers are favored for their ability to chemically crosslink into cell scaffolds and subsequently biodegrade. Moreover, OPF is a hydrophilic polymer that forms hydrogels when crosslinked, ideal for creating soft tissue scaffolds, and PPF is a hydrophobic polymer that forms stiff plastics, ideal for bone tissue. In this work, two fumarate-based polymers were investigated for tissue engineering applications. First, OPF was developed into a hydrogel platform that contains positively charged monomers and cell attachment biomolecules. This charged hydrogel platform was used to encapsulate valvular interstitial cells (VIC) to study how a positively charged environment affected the VIC phenotype. Second, poly(butylene fumarate) (PBF) was synthesized using 1,2-butanediol and 1,3-butanediol to make two variations of PBF, poly(1,2-butylene fumarate) (1,2-PBF) and
poly(1,3-butylene fumarate) (1,3-PBF). The chemical and material properties of 1,2-PBF and 1,3-PBF were then characterized for use as a bone scaffold.

Specific Aim 1: Fabricate a synthetic OPF-based hydrogel with tunable positively charged densities.

The first fumarate-based polymer explored was OPF. This polymer is made with poly(ethylene glycol) which gives OPF hydrophilic and bioinert properties. These properties are desired for making soft tissue models and reducing the number of environmental variables when testing cell-material interactions. Based on the properties, OPF was used as a base polymer to create a positively charged hydrogel for valvular engineering. In Chapter 3: “Acid Scavenger Free Synthesis of Oligo(Poly(Ethylene Glycol) Fumarate) Utilizing Inert Gas Sparging”, Tissue Engineering: Part C, 2021, 27(5), 296-306, OPF was synthesized in a one-step process that reduced the number of post-processing steps from tradition synthesis methods and produced higher molecular weight polymer.\(^2\) Nitrogen sparged OPF was then made into hydrogels with poly(ethylene glycol) diacrylate (PEGDA, \(M_n 575\)) and shown to have a modulus within the soft tissue range.

Outlined in Chapter 4: “Charged Oligo(Poly(Ethylene Glycol) Fumarate) Hydrogels for Encapsulating Valvular Interstitial Cells”, the OPF polymer platform was combined with a positively charged molecule [2-(methacryloyloxy)ethyl] trimethylammonium chloride (MAETAC), cell attachment biomolecules, and photoinitiators to make a synthetic hydrogel for valvular engineering.\(^5\)–\(^10\) To create hydrogels with varying concentrations of positively charged environments, different concentrations of MAETAC were photocrosslinked into the polymer network. Crosslinking analysis showed that the charged hydrogels supported varying concentrations of MAETAC up to 200 mM. Furthermore, a commonly used cell
attachment peptide (RGD) was tethered to a poly(ethylene glycol) molecule and photocrosslinked into the polymer network to increase cell attachment and long-term survival.\textsuperscript{11–16}

A gold standard for photocrosslinking hydrogels is Irgacure 2959 (I2959). Typically, it took 15-minutes of UV exposure to fully crosslink the charged hydrogels with I2959. To limit the amount of UV exposure time needed to crosslink the charged hydrogels, the water-soluble photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was explored.\textsuperscript{17} The LAP reduced the UV crosslinking time from 15-minutes to 3-minutes and was further used as the photoinitiator to make positively charged hydrogels. This work developed an OPF hydrogel platform with varying concentrations (0-200 mM) of positively charged monomers that can be UV crosslinked within 3-minutes.

Future iterations of the charged OPF hydrogel platform could incorporate negatively charged monomers, like sodium methacrylate, to make negatively charged cell environments. The versatility of the OPF hydrogel could support the addition of another material property that could play a role in calcific aortic disease progression. Specifically, substrate stiffness has been shown to cause VICs to differentiate into an osteoblastic phenotype.\textsuperscript{18} Combining substrate stiffness and environmental charge of the OPF hydrogels could create a 3D calcific diseases model for further cardiovascular research. Generally, OPF has the potential to be an ideal candidate for a tissue-specific drug-releasing hydrogel. Through the multiple unsaturated double-bonds, bioactive molecules can be functionalized into the OPF backbone and made into a hydrogel. Since the bioactive molecules are covalently bonded to the OPF, controlled release would depend on the degradation time of the polymer and tissue type.
Specific Aim 2: Characterize the viability of the valvular interstitial cells (VICs) when exposed to the positively charged OPF hydrogel solution, during UV encapsulation, and in culture through fluorescent assay analysis.

The positively charged OPF hydrogel was used to encapsulate VICs and test the cytotoxicity of the materials. Results of cytotoxicity studies are described in Chapter 4: “Charged Oligo(Poly(Ethylene Glycol) Fumarate) Hydrogels for Encapsulating Valvular Interstitial Cells”. Initial encapsulation studies showed that VICs did not survive the encapsulation process, regardless of the presence of the cell attachment biomolecule. The cytotoxicity of the base OPF hydrogel, without MAETAC, was then investigated. Three factors were considered as the reason for high cell mortality. The first factor was residual hydrochloric acid byproduct leftover from synthesizing OPF. It was found that OPF was still extremely acidic. Therefore, to neutralize the acidity the OPF hydrogel solution was reset to a pH of 7.4 with sodium hydroxide. Unfortunately, VICs still did not survive the encapsulation process so it was hypothesized another factor was also contributing to the cytotoxicity of the hydrogel.

The second factor investigated was the UV exposure time VICs had to endure during encapsulation. Originally, the OPF hydrogels were crosslinked with the photoinitiator I2959 which took 15-minutes to completely form the hydrogels. As previously stated, LAP was investigated as a faster photoinitiator and it reduced the UV exposure time VICs experienced from 15-minutes to 3-minutes. Again, this did not increase cell survival within the OPF hydrogels.

The final factor that was investigated as a contributor to VICs cytotoxicity was the polymers that made up the hydrogel solution and scaffolds. Specifically, a conditioned media
made from leached components of the hydrogel, the hydrogel solution VICs are suspended in before encapsulation, and the individual polymers (OPF, PEGDA) in a pH 7.4 phosphate buffer solution were tested for cytotoxicity. The conditioned media showed high cell viability indicating VICs were dying before encapsulation. The hydrogel solution (pH 7.4) containing the OPF, PEGDA, and LAP showed moderate cell viability, but extensive cell morphology changes and VICs were detaching from the plate surface. When the individual OPF components were tested OPF was found to have high cell viability and VICs remained attached to the plate surface but VICs exposed to the PEGDA (Mn 575) started to die. Upon further investigation into PEGDA’s cytotoxicity, it was found that VIC membranes started forming vesicles and detach from the plate surface. This indicates that PEGDA (Mn 575) was a major contributor to VIC cell death.\textsuperscript{19,20}

Future work should aim to improve VICs’ survival during encapsulation, specifically, addressing the OPF acidity and PEGDA crosslinker problems. It is suggested that OPF’s biocompatibility could be improved through a series of washing steps that would extract residual hydrochloric acid. Also, using a higher molecular weight PEGDA, around 3400 Da, has been shown to improve cell viability.\textsuperscript{19} Once VICs have been shown to survive in OPF hydrogels further cell studies could aim to test positively and negatively charged environments on encapsulated VICs to investigate how environmental charged affects VIC phenotype and if it is a contributing factor to calcific aortic disease progression.

\textit{Specific Aim 3: Investigate the chemical and material properties of 1,2-PBF and 1,3-PBF polymers and crosslinked films.}
The second fumarate-based polymer explored for tissue engineering was PBF. Poly(butylene fumarate) is a highly viscous hydrophobic polymer that creates stiff plastic materials that can hydrolytically degrade. In addition, PBF has the ability to be synthesized with different conformations of butylene which alter the chemical and material properties, making the polymer ideal for engineering biological interfaces that contain biomechanical gradients. Using the same acid scavenger free synthesis technique outlined in Chapter 3, PBF was synthesized with two types of butanediol to investigate the potential of new biomaterials for engineering the bone-ligament (BL) interface. The results of synthesizing 1,2-PBF and 1,3-PBF were described in Chapter 5: “Characterization and Development of Poly(1,2-Butylene Fumarate) and Poly(1,3-Butylene Fumarate) for Tissue Engineering”. The chemical and material analysis showed that 1,2-PBF and 1,3-PBF synthesized but did not contain the same degree of polymerization. The molecular weight of 1,2-PBF was considerably less than the 1,3-PBF but was found to have a more uniform distribution. It was hypothesized that the ethyl in the 1,2-butane group causes steric hindrance during polymerization, reducing the chain length of the polymer. Differences between the two polymers were also found in their respective glass transition (T_g) temperatures: 1,3-PBF had a lower T_g than 1,2-PBF. The difference in T_g can also be attributed to the butylene side groups and how easily the polymers pack together during phase transitions.

To create stiff crosslinked biomaterials 1,2-PBF and 1,3-PBF were mixed with a diethyl fumarate crosslinker and the photoinitiator phenylbis(2,4,6-trimethylbenzoyl) phosphine oxide (BAPO), and cast into films. The degree of crosslinking of the films was shown to be high for both PBF polymers. Moreover, 1,2-PBF was found to be more flexible than the 1,3-PBF films when flexed, indicating the differences, most likely in molecular
weights, between the 1,2-butylene and 1,3-butylene translate up to crosslinked films. It is hypothesized that the difference in the flexibility of the films is due to the side group associated with each PBF polymer and how they affect the crosslinking density of the films.

Future work will need to study the cytotoxicity of 1,2-PBF, in order for the polymers to be considered biomaterials, and compare biocompatibility to 1,3-PBF and PPF, as a biomaterial for bone engineering. Specifically, cytotoxicity should focus on relevant cell types, like osteoblasts or mesenchymal stems cells, to study the biomaterials potential as bone scaffolds. Also, a systematic comparative analysis between the chemical and material properties of 1,2-PBF, 1,3-PBF, 1,4-PBF, and PPF would offer insights into their utility in tissue engineering, creating a catalog of the chemical, materials, and biological properties for each polymer which could be useful for making scaffolds that mimic the biomechanical and biochemical gradients found in the bone-ligament interface. Finally, characterizing the viscosity of the PBF biomaterials is needed to develop them into 3D printable bioinks for making a bone-ligament construct.

**Future Work**

Since it was found that VICs did not survive when exposed to the OPF hydrogel solution due to the OPF acidity and PEGDA (Mn 575) some adaptions to the OPF synthesis and PEGDA type should be investigated to help improve biocompatibility. To increase the OPF biocompatibility, post-processing steps should be used to remove residual hydrochloric acid (HCl) from the OPF polymer. Implementing washing steps using a basic solution will neutralize the acid but there is a risk of losing OPF in the aqueous phase due to the polymer’s hydrophilic properties. Alternatively, filtering OPF in dichloromethane through a silica column could be used to separate the polar hydrochloric acid from the polymer, allowing for
isolation and collection of the purified OPF. A final precipitation step using ice-cold diethyl ether and vacuum drying would ensure that any residual organic solvents are completely removed and the OPF polymer is completely dry. Next, an investigation into the cytotoxicity of different molecular weights of PEGDA, ranging from 700 – 4,600 g/mole, on VICs should be run to understand which crosslinker has the highest cell viability. Additionally, an expanded cytotoxicity study comparing PEGDA molecular weight to the presence of the acrylate end-groups could delineate which property of PEGDA is causing VIC mortality. This can be done by exposing VICs to PEGDA or PEG with molecular weights ranging from 575 – 4,600 g/mole and then analyzing cell mortality through a fluorescent live/dead stain. Furthermore, altering the PEGDA chain length will change the mechanical properties of the charged OPF hydrogel. It is important that the charged OPF hydrogels have an elastic modulus within the soft tissue range (10-30 KPa) because VICs respond differently to substrates with differing mechanical stiffnesses. Therefore, a mechanical properties study through compression testing would outline the elastic modulus of charged OPF hydrogels with varying molecular weights of PEGDA. Results would catalog which PEGDA molecular weights create charged OPF hydrogels within the soft tissue range and are suitable for culturing VICs in charged OPF hydrogels.

Once the OPF hydrogel system can support VICs for an extended time frame (7-days), VICs can be exposed to positively charged environments by incorporation of MAETAC into the hydrogels, to test if positively charged environments cause an osteoblastic-like phenotype in VICs. Studies should focus on analyzing key osteoblastic markers such as calcium deposition from VICs into the extracellular matrix, expression of intracellular α-smooth muscle actin proteins, and up-regulation of characteristic gene
markers (α-smooth muscle actin, osteocalcin, and transforming growth factor-β1). To determine calcium deposition a histological analysis using Alizarin Red S staining can be used and immunohistochemistry can be used to fluorescent stain for α-smooth muscle actin formation within the cell which would identify cell morphology and α-smooth muscle actin production. Finally, through DNA isolation and real-time polymerase chain reaction analysis upregulation of the key osteoblastic gene markers in VICs grown in charged hydrogels can be used to determine if VICs have become osteoblastic-like. These experiments should increase the understanding of how charged biomaterials can affect the phenotype of VICs.

The next steps to develop 1,2-PBF and 1,3-PBF into a biomaterial for tissue engineering applications and bioprinting bone scaffolds includes determining the material’s capacity to culture cells on, followed by materials studies that optimize the printing parameters of PBFs’ bioinks. If both PBF bioinks are to be considered biomaterials they must be able to support cell growth. To confirm biocompatibility cell attachment and viability studies will need to be run. Additionally, since PBFs are being developed for bioprinting bone scaffolds, osteoblasts should be used to determine cell attachment and growth on the materials. To study the biocompatibility of the materials a fluorescent live/dead and metabolic assay can be used, followed by actin and myosin immunohistochemical staining and cell attachment assays to determine osteoblast’s ability to attach and spread on the PBF materials. Once osteoblasts have been shown to survive on the materials, then an investigation into phenotypic expression of cultured osteoblasts can be run. Specific cell expressions that should be studied for osteoblasts are the production of extracellular products, like calcium deposition, through an Alizarin Red S stain to understand if 1,2-PBF and 1,3-PBF stimulate bone formation. Furthermore, genetic studies using real-time
polymerase chain reaction can be run to determine the up-regulation of osteoblastic genes used in bone remodeling, like alkaline phosphatase, type I collagen, osteopontin, and osteocalcin.

Upon determining that 1,2-PBF and 1,3-PBF can support osteoblast growth and proper phenotypic expression, 3D bioprinting studies should be run to outline parameters needed to print 1,2-PBF and 1,3-PBF bioinks into 3D porous scaffolds. Initially, a study of the viscosity of each bioink using rheometry will have to be run to help determine fluid flow properties during printing parameter optimization. Printing parameters such as the size and shape of the printing nozzle, extrusion pressure, printing speed and height, and UV curing time will need to be outlined in order to print high-resolution 3D scaffolds. To determine the size and shape of the printing nozzle and extrusion pressure a factorial design can be set up to systematically test the bioinks’ ability to flow through each nozzle type with increasing amounts of extrusion pressures. Successful nozzles and extrusion pressures will allow a constant stream of bioink flows. Next, printing speed, height, and UV curing time can be tested by printing lines of bioinks at varying print speeds, heights, and UV curing times. The printed lines then can be analyzed through microscopic imaging and software analysis of the line’s geometry. Successful printing speeds, heights, and UV curing times will result in an intact line with a tubular geometry, while a line that missing sections and has a flattened geometry will indicate a fast-printing speed, low printing height, or inadequate UV curing. Finally, after printing parameters have been optimized 3D porous scaffolds can be printed with 1,2-PBF and 1,3-PBF and the architecture of the scaffolds can be analyzed. Specifically, the scaffolds’ strut shape, spacing, and pore dimensions can be measured through microscopic imaging and software analysis. The strut shape, distance, and pore circularity
will help determine the print resolution of the PBF scaffolds. By characterizing the PBFs’ ability to grow osteoblasts and outlining printing parameters, 1,2-PBF and 1,3-PBF bioinks can be printed into 3D porous bone scaffolds for future bone tissue engineering applications.

**Broader Impact**

Overall, this work highlights two uses of fumarate-based polymers for tissue engineering applications. First, using OPF it was shown that a positively charged hydrogel system has the potential for valvular engineering. Further development of the charged OPF hydrogel system will aid in studying cell-material interactions of environment charge on VIC phenotype. This will further increase the current understanding of how the extracellular environments affect the progression of calcific valvular disease and aid in treatment development.

This work also reflects the investigation of chemically engineered PBF as a biomaterial for replicating the bone-ligament interface. New biomaterials are needed to help replicate complex systems found in the body such as the bone-ligament enthesis. As such, 1,2-PBF has never been investigated as a biomaterial for tissue engineering applications and has the potential of being a biomaterial for bone and osteochondral tissue. Moreover, PBF has the potential to be a bioprintable material for printing composite scaffolds that replicate the bone-ligament interface. Variations of PBF are a newer class of fumarate-based polymer and possibly can be used in 3D cell modeling and fabrication of a complex bone-ligament-bone medical device.

Finally, this work outlines the uses of fumarate-based polymers as tissue-engineered scaffolds for valvular research and bone tissue. Further research into current fumarate-based polymers offers an ability to make both soft and hard tissues that have tunable biomechanical
and biochemical gradients, ideal for replicating complex tissue interfaces. These polymers also have applications in additive manufacturing which extends past traditional scaffold processing techniques, like casting, into detailed scaffold platforms that mimic the tissue architecture. Further research and application of fumarate-based polymers show their utility not only in the tissue engineering and regenerative medicine field but also as biomaterials for other additive manufacturing applications, cell and organ modeling, and medical device development.
References


