RICE UNIVERSITY

Controlled Release of Osteogenic Factors from Injectable Biodegradable Composite Materials for Bone Tissue Engineering

by

Elizabeth LeBleu Hedberg

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

Doctor of Philosophy

APPROVED, THESIS COMMITTEE:

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

Kyriacos A. Athanasiou, Professor Bioengineering

Michael C. Gustin, Associate Professor Biochemistry and Cell Biology

John A. Janssen, Department Chair, Professor Biomaterials, University Medical Center, Nijmegen, The Netherlands

Alan W. Yasko, Chief, Section Chief Orthopaedic Oncology, Professor Surgery, University of Texas M. D. Anderson Cancer Center

Michael J. Yaszemski, Associate Professor Bioengineering and Orthopedics, Mayo Clinic College of Medicine, Rochester, MN

HOUSTON, TX

APRIL 2004
INFORMATION TO USERS

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

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

UMI Microform 3122477
Copyright 2004 by ProQuest Information and Learning Company. All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

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

Controlled Release of Osteogenic Factors from Injectable Biodegradable Composite Materials for Bone Tissue Engineering

by

Elizabeth LeBleu Hedberg

Composite materials based on the synthetic polymer poly(propylene fumarate) (PPF) were developed and characterized for use as porous controlled release scaffolds in bone tissue engineering. Through the use of poly(DL-lactic-co-glycolic acid) (PLGA) microparticles, the osteogenic peptide TP508 (Chrysalin®) was incorporated into the polymer phase of PPF-based scaffolds, creating a composite material that could act as a scaffold for guided tissue ingrowth as well as a vehicle for targeted drug delivery. Alteration of formulation parameters such as the TP508 loading of the microparticles, the microparticle to PPF ratio, and the initial leachable porogen content lead to variation in the release kinetics of the incorporated peptide in vitro. Inclusion of the microparticles into the scaffolds as well as changes in the scaffold formulation parameters did not alter the scaffolds in vitro degradation profile through 26 weeks.

Using results from the in vitro studies, two distinct release kinetic profiles were selected for further evaluation in vivo. Composite formulations exhibiting either a large initial burst release or a minimal initial burst release of 200 μg TP508 were implanted in 15.0 mm segmental defects in rabbit radii. Radiography, micro-computed tomography, and histomorphometry were used to elucidate the effect of varied release kinetics on bone formation at 12 weeks post-operative. Results showed that composite scaffolds exhibiting a large burst release of TP508 resulted in the greatest amount of bone. Analysis showed that bone formation was characterized by growth both into the pores of
the scaffold as well as guided across the defect along the surface of the implant. Further investigation revealed minimal degradation of the polymer after 18 weeks in vivo.

The studies presented here demonstrate the potential of PPF/PLGA composite materials for use in bone tissue engineering. These composite scaffolds offer controlled, targeted delivery of bioactive molecules as well as structural support for cellular infiltration and bone formation within osseous defects.

Additional work was conducted in the area of controlled release of polysaccharide oligomers. Initial experiments established that hyaluronan oligomers could be incorporated into PLGA microparticles and that parameters including PLGA molecular weight, hyaluronan molecular weight, and hyaluronan loading influenced the oligomer release kinetics.
ACKNOWLEDGEMENTS

Dedicated to the memory and spirit of my mother,
Floetta Mary LeBleu Hedberg

I miss you.

A thesis is truly a collaborative effort, and over the past six years I have had the privilege of working with a number of great individuals from a number of different laboratories around the world. For all these opportunities, I have to thank my advisor, Dr. Tony Mikos. Your support and guidance over the years have been invaluable, and I am honored to have been your student. I would also like to thank the members of my thesis committee, Drs. Kyriacos Athanasiou, John Jansen, Michael Gustin, Alan Yako, and Michael Yaszemski for their guidance and encouragement.

Chrysalis, Biotechnologies, Inc. supplied me with the peptide that was the focus of the majority of my studies. I would like to express my gratitude to Darrell Carney, Dennis McWilliams, Roger Crowther, and Andrew Tang for trusting with something so important; I tried to do you proud. I would also like to thank Jim Ryaby from Orthologic for joining in on the discussions.

The members of the Mikos lab have been there for me consistently through the years. I would like to express my sincere appreciation to them for sharing their knowledge in the lab and befriending me outside of it. I would also like to thank my undergraduate assistant, Charlie Shih. He gave me the opportunity to teach and, in the end, taught me so much.

During the later part of my studies I was invited to work in the lab of Dr. John Jansen at the University of Nijmegen Medical Center in the Netherlands. I was there for almost a year and I will never be able to express how grateful I am for having been given this opportunity and how much I learned. I would like to thank all the members of the
department, particularly Olga, Anja, Jacky, Jan-Paul, and the members of the
“International Research Office” for their technical assistance, friendship, and guidance in
all things Dutch.

Words cannot express my deep gratitude to the many friends who have
supporting me through all that has come my way over the past six years. I would like to
particularly acknowledge the other members of the “Fab Four,” Andi, Johnna, and
Rachael, who held me up when I was not strong enough to do it myself and never
allowed me to fall behind. I thank them for always being there for me, each in their own
way.

My family has faced a lot of difficulties during my tenure at Rice and we have
emerged stronger and closer than ever. I would have been lost without their constant
belief in me and my abilities. Their council, usually given in the middle of the night,
helped me focus and remember what was important.

Finally, I would like to thank my future husband, Shawn Dirk. He has made my
life complete.
TABLE OF CONTENTS

Title Page i
Abstract ii
Acknowledgements iv
Table of Contents vi
List of Tables ix
List of Figures x
Abbreviations xvii

Chapter I: Introduction: Bone Augmentation and Repair 1

Chapter II: Objectives 14

PART I: Poly(Propylene Fumarate)-Based Composite Materials

Chapter III: Controlled Release of an Osteogenic Peptide from Biodegradable Polymeric Composites
A. Abstract 15
B. Introduction 16
C. Materials and Methods 20
D. Results 27
E. Discussion 30
F. Conclusions 34
G. Tables and Figures 36

Chapter IV: In Vitro Degradation of Porous Poly(Propylene Fumarate)/Poly(DL-Lactic-co-Glycolic Acid) Composite Scaffolds
A. Abstract 46
B. Introduction 47
C. Materials and Methods 49
D. Results 59
E. Discussion 63
F. Conclusions 64
G. Tables and Figures 65

Chapter V: Bone Regenerative Effect of Varied Release Kinetics of the Osteogenic Peptide TP508 from Biodegradable Polymeric Scaffolds
A. Abstract 79
B. Introduction 80
C. Materials and Methods 84
D. Results 90
E. Discussion 93
F. Conclusions 96
G. Tables and Figures 97
Chapter VI: A Comparative Analysis of Radiography, Micro-Computed Tomography, and Histology for Bone Tissue Engineering
A. Abstract 108
B. Introduction 109
C. Materials and Methods 111
D. Results 114
E. Discussion 117
F. Conclusions 121
G. Tables and Figures 122

Chapter VII: In Vivo Degradation of Porous Poly(Propylene Fumarate)/Poly(DL-Lactic-co-Glycolic Acid) Composite Scaffolds
A. Abstract 131
B. Introduction 131
C. Materials and Methods 133
D. Results 135
E. Discussion 136
F. Conclusions 139
G. Tables and Figures 140

Chapter VIII: Bone Regeneration in Segmental Defects in the Rabbit Radius
A. Introduction 144
B. Materials and Methods 146
C. Results 151
D. Discussion 154
E. Tables and Figures 157

PART II: Preliminary Characterization of Additional Delivery Vehicles

Chapter IX: Controlled Release of Hyaluronic Acid Oligomers from Biodegradable Polymeric Carrier
A. Abstract 167
B. Introduction 168
C. Materials and Methods 170
D. Results 174
E. Discussion 175
F. Conclusions 180
G. Tables and Figures 181
Chapter X: rhBMP-2 Release from Injectable Poly(DL-Lactic-co-Glycolic Acid)/Calcium Phosphate Cement Composites
   A. Abstract  
   B. Introduction  
   C. Materials and Methods  
   D. Results  
   E. Discussion  
   F. Conclusions  
   G. Tables and Figures  

Chapter XI: References
## LIST OF TABLES

1-1: Scaffold design parameters 13

3-1a: Microparticle experimental design: high and low values for parameters tested in the Resolution III, two-level fractional factorial design 36

3-1b: Microparticle experimental design: combinations of the experimental values in the Resolution III, two-level fractional factorial design 36

3-2a: PPF composite experimental design: combinations of the experimental values in the Resolution III, two-level fractional factorial design 37

3-3: Entrainment efficiencies of TP508 in PLA/PEG microparticles 38

4-1a: PPF/PLGA degradation experimental design: combinations of the experimental values in the Resolution III, two-level fractional factorial design 65

4-2a: PPF/PLGA degradation experimental design: high and low values for parameters tested in the Resolution III, two-level fractional factorial design 65

5-1: Classes of poly(propylene fumarate)/poly(D,L-lactic-co-glycolic acid) controlled release scaffolds 97

5-2: Histological grading scale 98

5-3: Bone penetration values 99

6-1: Histological grading scale 122

6-2: Quantitative results of all samples 123

7-1: Properties of PPF/PLGA composite scaffold classes 140

7-2: Histological grading scale 140

8-1: Implant formulations 157

8-2: Histological grading scale 157

9-1a: Microparticle experimental design: high and low values for parameters tested in the $2^{4-1}$ fractional factorial design 181

9-1b: Microparticle experimental design: combinations of the experimental values in the $2^{4-1}$ fractional factorial design 181
LIST OF FIGURES

3-1: Cumulative release kinetics of TP508 from PLGA/PEG microparticles into PBS at 37°C with agitation (70 rpm) expressed as normalized mass release: the cumulative normalized mass released for all time points; (B) the cumulative normalized mass released on days 1, 4, 14, and 28; error bars represent means ± standard deviation for n=3.

3-2: Main effects of TP508 loading and PEG content on normalized cumulative mass of TP508 released from PLGA/PEG microparticles for days 1, 4, 14, 28. A positive number indicates that the particular parameter had an increasing effect on the normalized cumulative mass released as the value was changed from a low (L) level (-) to a high (H) level (+) [see Table 3-1 (a)]. Error bars represent the standard errors of the effect (day 1, 0.01; day 4, 0.03; day 14, 0.01; day 28, 0.02).

3-3: Cumulative release kinetics of TP508 from PPF composite scaffolds into PBS at 37°C with agitation (70 rpm): (A) the cumulative normalized mass released for all time points; error bars are excluded for clarity. (B) the cumulative normalized mass released on days 1, 4, 14, and 28; error bars represent means ± standard deviation for n=3.

3-4: Main effects of microparticle TP508 loading, microparticle PEG content, scaffold polymer microparticle loading, and scaffold weight percent of NaCl on normalized cumulative mass of TP508 release from PPF composite scaffolds for days 1, 4, 14, and 28. A positive number indicates that the particular parameter had an increasing effect on the normalized cumulative mass released as the value was changed from a low (L) level (-) to a high (H) level (+) [see Table 3-2 (a)]. A negative number indicates a decrease in the normalized cumulative mass release as the parameter was changed from low (L) level (-) to a high (H) level (+). Error bars represent the standard errors of the effect (day 1, 0.006; day 4, 0.007; day 14, 0.01; day 28, 0.009).

3-5: Day 1 normalized cumulative mass of TP508 released from PLGA/PEG microparticles and the PPF composites into which the PLGA/PEG microparticles were embedded. PPF composite formulations exhibited statistically lower normalized cumulative TP508 mass release (p<0.05) than the corresponding non-embedded microparticles. Error bars represent means ± standard deviation for n=3.

3-6: Cumulative release kinetics of TP508 from PPF composite scaffolds into PBS at 37°C with agitation (70 rpm) expressed as cumulative mass released per scaffold volume: (A) the cumulative mass released for all time points; error bars are excluded for clarity. (B) the cumulative mass released on days 1, 4, 14, and 28; error bars represent means ± standard deviation for n=3.
3-7: Main effects of microparticle TP508 loading, microparticle PEG content, scaffold polymer microparticle loading, and scaffold weight percent of NaCl on cumulative mass of TP508 released from PPF composite scaffolds for days 1, 4, 14, and 28. A positive number indicates that the particular parameter had an increasing effect on the normalized cumulative mass released as the value was changed from a low (L) level (-) to a high (H) level (+) [see Table 3-2(a)]. A negative number indicates a decrease in the normalized cumulative mass released as the parameter was changed from the low (L) level (-) to a high (H) level (+). Error bars represent the standard errors of the effect (day 1, 0.009; day 4, 0.009; day 14, 0.01; day 28, 0.01).

4-1: Deviation of solution pH from buffer Ph blanks as a function of degradation time for PPF/PLGA composite scaffolds (C1, C2, C3, C4) and unloaded PPF scaffolds (B1, B2) as determined by micro-computed tomography. Results are presented as means ± standard deviation for n = 3.

4-2: Fractional change in polymer mass during in vitro degradation for PPF/PLGA composite scaffolds (C1, C2, C3, C4) and unloaded PPF scaffolds (B1, B2). Results are presented as means ± standard deviation for n = 4.

4-3: Fractional change in length during in vitro degradation for PPF/PLGA composite scaffolds (C1, C2, C3, C4) and unloaded PPF scaffolds (B1, B2). Results are presented as means ± standard deviation for n = 4.

4-4: Fractional change in diameter during in vitro degradation for PPF/PLGA composite scaffolds (C1, C2, C3, C4) and unloaded PPF scaffolds (B1, B2). Results are presented as means ± standard deviation for n = 4.

4-5: Sample water absorption during in vitro degradation for PPF/PLGA composite scaffolds (C1, C2, C3, C4) and unloaded PPF scaffolds (B1, B2). Results are presented as means ± standard deviation for n = 4.

4-6: Compressive modulus and peak stress during in vitro degradation for PPF/PLGA composite scaffolds (C1, C2, C3, C4) and unloaded PPF scaffolds (B1, B2). Results are presented as means ± standard deviation for n = 5.

4-7: Porosity during in vitro degradation for PPF/PLGA composite scaffolds (C1, C2, C3, C4) and unloaded PPF scaffolds (B1, B2) as determined by micro-computed tomography. Results are presented as means ± standard deviation for n = 3.

4-8: μCT images of PPF/PLGA scaffolds at day 4 and week 26. (a.) C1, day 4; (b.) C2, day 4; (c.) C3, day 4; (d.) C1, week 26; (e.) C2, day 26; (f.) C3, day 26. Sample area was 4 mm in diameter and 2.5 mm in thickness with a special resolution of 10 μm. Bars represent 1 mm.
4-9: Porosity during in vitro degradation for PPF/PLGA composite scaffolds (C1, C2, C3, C4) and unloaded PPF scaffolds (B1, B2) as determined by mercury intrusion porosimetry. Results are presented as means ± standard deviation for n = 3.

4-10: Scanning electron micrographs of porous PPF/PLGA composite scaffolds. C1, day 4 (a) and week 26 (b); C2, (d) and week 26 (d); C3, (e) and week 26 (f); C4, (g) and week 26 (h).

4-11: Scanning electron micrographs of PLGA microparticles embedded in PPF (formulation C4) at day 4 (a) and week 26 (b).

5-1: Cumulative mass release of TP508 from PPF composite scaffolds represent means ± standard deviation for n=3. Adapted from [20].

5-2: Histological sections of PPF/PLGA scaffolds. (a) Bone ingrowth into and guidance around PPF/PLGA scaffold (class LB200); (b,c) pore bone fill, active bone formation, bone/implant contact (class LB200); (d) scaffolds with no bone ingrowth exhibiting bone growth around scaffold (guided growth; class MB200); (e) cartilage formation (class MB200).

5-3: Histological sections of PPF/PLGA scaffolds. (a, b) Fibrous tissue, collagen fibers, blood vessels (classes NR and LB200); (c,d) polymer fragmentation with increased inflammatory response (classes MB100 and MB200); (e) cross-sectional section with no ingrowth but extensive growth around scaffold (guided growth; class LB200).

5-4: Results of histological scoring of longitudinal sections for bone growth into defect at defect borders (bridging). Results are means ± standard deviation for n=5.

5-5: Results of histological scoring of longitudinal sections for bone growth around the outside of the implant (guided-growth). Results are means ± standard deviation for n=5.

5-6: Results of histological scoring for tissue response within the pores of the implant. (A.) results of analysis of longitudinal sections; (B) results of analysis of lower cross-sectional sections (□) and upper cross-sectional sections (□). Results are means ± standard deviation for n=5.

5-7: Results of histological scoring for polymer degradation. (A) Results of analysis of longitudinal sections; (B) results of analysis of lower cross-sectional sections (□) and upper cross-sectional sections (□). Results are means ± standard deviation for n=5.

5-8: Average maximum distance of bone penetration into the polymer scaffold for each scaffold class. Results are means ± standard deviation for n=5.
6-1: Schematics used to guide investigators in the quantitative evaluation of the radiographs. (a) Radiographic evaluation of the degree of union. Percentage values refer to the percent of the given defect border that is filled with bone that spans the defect. (b) Radiographic evaluation of the bone formation in the defect. Percentage values refer to the percent of the defect area that is filled with bone.

6-2: Representative radiographs from each scaffold classes taken 12 weeks post-operatively. (a) LB200, (b) MB200, (c) MB100, and (d) NR.

6-3: Results from radiographic scoring for the amount of bone formation within the defect area (a) and, the amount of bridging of one defect end with the other (b). Results represent means ± standard deviation for n = 5.

6-4: Radiographs of representative sample for Group A. Radiograph in (a) was taken prior to sacrifice at 12 weeks post-operatively; radiograph in (b) was taken after embedding of the sample on methylmethacrylate.

6-5: μCT reconstructions of representative sample for Group A; (a) position 1; (b) rotated 180°; (c) longitudinal section; cross-sectional section.

6-6: Histological sections from representative sample from Group A; (a,b) cross-sectional sections; (b,c) longitudinal sections of the implant. Bone overgrowth can be observed on the top of the implant samples in c and d.

6-7: Radiography (a), microCT (b), and histology (c,d) images from representative sample from Group B.

7-1: Histological sections of PPF/PLGA scaffolds. (a,b) Degradation was characterized by micro-fragmentation of the cross-linked network and was associated with an increase in inflammatory response; (c) multinucleated giant cells in the pores of the polymer scaffold; (d) tissue ingrowth was evident in circular pores previously occupied by PLGA microparticles.

7-2: Histological sections of PPF/PLGA scaffolds. (a,b,c) Bone ingrowth into pores of PPF/PLGA scaffold; (d) osteoblastic front inside a pore of the scaffold; (e) cartilage formation.

7-3: Results of histological scoring for polymer degradation (a) and tissue response within the pores of the implant (b) at 12 and 18 weeks. Results are means ± standard deviation for n=5.

8-1: Schematics used to guide investigators in the quantitative evaluation of the radiographs. (a) Radiographic evaluation of the degree of union. Percentage values refer to the percent of the given defect border that is filled with bone that spans the defect. (b) Radiographic evaluation of the bone formation in the defect. Percentage values refer to the percent of the defect area that is filled with bone.
8-2: Results from radiographic scoring for the amount of bone formation within the defect area (a) and the amount of bridging from one defect border to the other. Results represent means ± standard deviation for n=5.

8-3: Three-dimensional reconstructions of the 5 mm region of interest in the center of the defect. (a) PPF-200, (b) PPF-NR, (c) GEL-200 and, (d) GEL-NR.

8-4: Volume of bone per unit length (mm³/mm) for the region of interest as determined by μCT. Results are presented as means ± standard deviations for n=3.

8-5: Histological sections. (a,b) Bone growth around a PPF implant; (b) evidence of bone formation (osteoblasts, osteoclasts, and osteocytes) in the pores a PPF scaffold; (c) fibrous tissue with minimal inflammation in the pores of the polymer scaffold, (a, d) vasculature, (e) inflammation observed in areas of polymer degradation, and (e) typical structure of bone formation in microparticle/Pluronic gel constructs.

8-6: Quantitative results of histological analysis using the scoring system presented in Table 8-3. (a) Bridging of the defect border, (b) bone growth guided around PPF scaffold, (c) tissue response in the pores of the scaffold and, (d) polymer degradation. Results are presented as means ± standard deviations for n=5.

8-7: Average maximum distance of bone penetration into the polymer scaffold for the two polymer scaffold formulations. Results are means ± standard deviation for n=5.

8-8: Area of bone per unit length (mm²/mm) for the region of interest as determined by μCT. Results are presented as means ± standard deviations for n = 3.

9-1: Entrapment efficiency of HY oligomers within PLGA microparticles using an established ((water-oil)-water) technique. The results are presented as means ± standard deviation for n = 3.

9-2: Main effects of PEG content, HY loading, HY molecular weight, and PLGA molecular weight on HY entrapment efficiency within PLGA microparticles. A positive number indicates an increase in the entrapment efficiency with a change from the low level to high level for the individual parameters and a negative number indicates a decrease in the rate of release. The results are presented as means ± standard error of effect for n = 3.

9-3: Cumulative release of HY from PLGA/PEG microparticles in PBS at 37°C. The release is expressed as normalized mass release for all time points. The results are presented as means ± standard deviation for n = 5.
9-4: Rate of release of HY from the PLGA/PEG microparticles during the 1-day burst release period. The results are presented as means ± standard deviation for n = 5.

9-5: Main effects of the PEG content, HY loading, HY molecular weight, and PLGA molecular weight on the rate of release during the first day. A positive number indicates an increase in the rate of release with a change from the low level to high level for the individual parameters and a negative number indicates a decrease in the rate of release. The results are presented as means ± standard error of effect for n = 5.

9-6: Rate of release of HY from the PLGA/PEG microparticles in PBS during the day 1 through day 14 time period. The results are presented as means ± standard deviation for n = 5.

9-7: Main effects of the PEG content, HY loading, HY molecular weight, and PLGA molecular weight on the rate of release period during the day 1 through day 14 time period. A positive number indicates an increase in the rate of release with a change from the low level to high level for the individual parameters and a negative number indicates a decrease in the rate of release. The results are presented as means ± standard error of effect for n = 5.

9-8: Rate of release of HY from the PLGA/PEG microparticles in PBS during the day 14 through day 28 time period. The results are presented as means ± standard deviation for n = 5.

9-9: Main effects of the PEG content, HY loading, HY molecular weight, and PLGA molecular weight on the rate of release during the day 14 through day 28 time period. A positive number indicates an increase in the rate of release with a change from the low level to high level for the individual parameters and a negative number indicates a decrease in the rate of release. The results are presented as means ± standard error of effect for n = 5.

10-1: Scanning electron micrograph of PLGA microparticles, showing the spherical shape of the microparticles. Bar represents 10 μm.

10-2: Micro-computed tomography of 30/70 wt% PLGA/Ca-P cement composite. In the μCT reconstruction, PLGA microparticles appear as pores in the Ca-P cement. Bar represents 1 mm.

10-3: Scanning electron micrograph of 30/70 wt% PLGA/Ca-P cement composite. Several different sized microparticles surrounded by micro-porous Ca-P cement can be observed. Bar represents 20 μm.

10-4: Results of mechanical testing of Ca-P cement and PLGA/Ca-P cement composite. Error bars represent means ± standard deviation for n=5. The asterisk indicates a significant difference between both groups (p<0.001).
10-5: Results of LIVE-DEAD cytotoxicity assay of Ca-P cement. Normalized viability, based on total fluorescence, is displayed for cells exposed to unconditioned medium (control) and cells exposed to conditioned medium in different dilutions. Error bars represent means ± standard deviation for n=3. Variation among LIVE bar means is not significantly greater than expected by chance (p> 0.05).

10-6: *In vitro* release of rhBMP-2 loaded PLGA microparticles and PLGA/Ca-P cement composite in pH 7.4 and pH 4.0 under continuous shaking at 37° C. Error bars represent means ± standard deviation for n=4.
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>3-D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ASTM</td>
<td>American Society for Testing and Materials</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>BP</td>
<td>benzoyl peroxide</td>
</tr>
<tr>
<td>C_p</td>
<td>mass fraction polymer</td>
</tr>
<tr>
<td>C_s</td>
<td>mass fraction NaCl</td>
</tr>
<tr>
<td>CaCO_3</td>
<td>calcium carbonate</td>
</tr>
<tr>
<td>CH_2Cl_2</td>
<td>methylene chloride</td>
</tr>
<tr>
<td>CO_2</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>d_d</td>
<td>diameter of scaffold after drying</td>
</tr>
<tr>
<td>d_i</td>
<td>initial diameter of scaffold</td>
</tr>
<tr>
<td>d_w</td>
<td>diameter of scaffold while wet</td>
</tr>
<tr>
<td>l_d</td>
<td>length of scaffold after drying</td>
</tr>
<tr>
<td>l_i</td>
<td>initial length of scaffold</td>
</tr>
<tr>
<td>l_w</td>
<td>length of scaffold while wet</td>
</tr>
<tr>
<td>DCPA</td>
<td>dicalcium phosphate anhydrous</td>
</tr>
<tr>
<td>DdH_2O</td>
<td>distilled, deionized water</td>
</tr>
<tr>
<td>DMT</td>
<td>N,N-dimethyl-p-toluidine</td>
</tr>
<tr>
<td>GPC</td>
<td>gel permeation chromatography</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronic acid</td>
</tr>
</tbody>
</table>
Hap       hydroxyapatite
HCl       hydrochloric acid
I$^{125}$  radioactive iodine
$m_d$     mass of scaffold after drying
$m_{i,p}$  initial polymer mass
$m_{s,i}$  initial scaffold mass
$m_w$     mass of scaffold while wet
$M_W$     weight average molecular weight
$M_n$     number average molecular weight
$n$       number of repetitions
NaCl      sodium chloride
NaOH      sodium hydroxide
OP-1      osteogenic protein-1
PBS       phosphate buffered saline
PEG       poly(ethylene glycol)
PGA       poly(glycolic acid)
PVA       poly(vinyl alcohol)
PI        polydispersity index
PLA       poly(lactic acid)
PLGA      poly(DL-lactic-co-glycolic acid)
PMMA      poly(methyl methacrylate)
PPF       poly(propylene fumarate)
PPF-DA    poly(propylene fumarate)-diacrylate
rhBMP-2   recombinant human bone morphogenetic protein-2
TGF-β     transforming growth factor β
TP508     23 amino acid petide with the trade name Chrysalin$^\circledR$
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{por}$</td>
<td>pore volume</td>
</tr>
<tr>
<td>$\text{ZnCl}_2$</td>
<td>zinc chloride</td>
</tr>
<tr>
<td>$\alpha$-TCP</td>
<td>tricalcium phosphate</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>porosity</td>
</tr>
<tr>
<td>$\mu$CT</td>
<td>micro-computed tomography</td>
</tr>
<tr>
<td>$\rho$</td>
<td>density (g/mL)</td>
</tr>
<tr>
<td>$\rho_s$</td>
<td>density of NaCl (2.17 g/mL)</td>
</tr>
<tr>
<td>$\rho_p$</td>
<td>density of polymer (1.28 g/mL)</td>
</tr>
</tbody>
</table>
CHAPTER I

Background: Bone Augmentation and Repair*

1. Introduction

Bone is a complex tissue that performs a variety of integral functions in the maintenance of the body’s many systems. Bone is the primary reservoir of calcium, phosphate, and other important ions and it acts to regulate the concentration of these minerals throughout the body. In addition, the marrow found within the bone generates red and white blood cells for immunoprotection and oxygenation of other tissues. Finally, bone provides structural support and protection and sites of muscle attachment for locomotion [1]. Under normal conditions, bone has the ability to repair and regenerate itself without the formation of a scar. Under certain circumstances, however, the body’s own functions fail and intervention is required.

This chapter begins with an overview of the structural properties and natural repair mechanism of bone. It then focuses on the current clinical methods and materials used for bone augmentation and repair. Finally, it addresses the development of synthetic, bioresorbable polymers for use as scaffolds for cell transplantation or conduits for guided tissue in-growth.

2. Bone Physiology

Mature bone is a highly vascularized tissue composed of cells and an extracellular matrix (ECM). The cells include bone forming cells (osteoblasts), bone resorbing cells (osteoclasts), and bone maintaining cells (osteocytes). The ECM is a

composite material consisting of organic and inorganic components. The organic component, also called the osteoid, is secreted by the osteoblasts. The osteoid constitutes approximately 50% of bone by volume (25% by weight) and is composed of 90% Type I collagen and 10% amorphous ground substance (primarily glycoproteins and glycosaminoglycans) [2]. The organic component gives bone its form and contributes to its ability to resist tension [3]. The inorganic component is a mineral phase composed of calcium crystals primarily in the form of hydroxyapatite ([Ca₁₀(PO₄)₆(OH)₂]. This component makes up 50% of bone by volume (75% by weight) [2] and gives bone most of its stiffness and strength [3].

When bone is initially laid down, it is structurally weak and unorganized [1]. Within days, the primary bone is remodeled to become lamellar bone. Lamellar bone has alignment of the collagen fibers and mineral phase. Mature lamellar bone exists as cortical (also called compact) or trabecular (cancellous, spongy) bone. Cortical bone is found on the outer surface of most bones. It is dense with respect to trabecular bone (apparent density equal to 1.8 g/cm³ for cortical versus 0.1 - 1.0 g/cm³ for trabecular), and contains closely spaced groups of concentric lamellar bone rings called osteons [1]. Cortical bone functions mechanically in tension, compression, and torsion [3]. Trabecular bone occurs near the ends of long bones and in the center of small and flat bones. It is composed of an array of plates and rods that form an open cell foam [1]. Trabecular bone functions mainly in compression [3].

Bone has the unique ability to remodel itself along lines of stress in response to injury or other changes in its mechanical environment [4]. When a bone is fractured, the body creates new bone to connect the broken fragments, and then remolds the new bone to optimize its mechanical function in the particular region of the skeleton where the fracture has occurred.
When a bone fractures, the repair process begins immediately [1]. The broken blood vessels in the vicinity of the fracture fill the space in and around the fractured bone ends with a fracture hematoma. New blood vessels grow into the defect, and bone forming cells migrate into the area. The cells (osteoblasts) secrete the osteoid, which will become mineralized and eventually become woven bone. Woven bone is isotropic, and will be remodeled by the sequential action of removal and redeposition along stress lines. Remodeling occurs continuously in the body, and results in the anisotropic structure of lamellar bone that is mechanically and structurally optimized for each area of the skeleton [5].

As many as 5 to 10% of the 6.2 million fractures sustained by patients in the United States each year result in fracture nonunion and decreased mechanical support [6]. When this occurs, an attempt must be made to induce bone regeneration for skeletal repair or reconstruction. The surgeon strives to make local skeletal conditions mimic those that the body would expect to encounter during normal fracture repair. In essence, he or she attempts "trick" the body into initiating the fracture repair cascade [5].

3. Current Techniques

The current gold standard for bone augmentation and repair is the trabecular bone autograft. Transplantation of autologous tissue involves the harvesting of bone from a distant location and implanting it into the defect site. A similar approach uses bone harvested from cadavers (allogeneous tissue). Both techniques are aimed at the ultimate restoration of bone tissue. Transplantation provides a scaffold similar to the surrounding bone upon which the patient's bone can invade, lay down extracellular matrix, and remodel. Although these treatments are often successful, they have their associated problems and limitations. Autologous tissue is limited in supply, is often not available in the shape needed, and its relocation leaves a secondary defect site.
Allogenous tissue runs the risk of pathogen transfer and immune system rejection of the foreign material. Both transplantation techniques injure local vessels and leave bleeding host bone at the defect site which will activate the coagulation cascade and may lead to necrosis of the grafted tissue [1].

Poly(methyl methacrylate) (PMMA) is a non-degradable bone cement that is widely used to fill bone defects or provide fixation of prosthesis to bone. PMMA is a biologically inert material and acts as a permanent barrier preventing fracture healing or integration with host bone. A recent study of twenty-two commercially available plain PMMA based bone cements reports compressive strengths ranging from 75 to 115 MPa and moduli of elasticity from 1700 to 3100 MPa [7]. Because these values are much greater than those of trabecular bone (5-10 MPa and 50-100 MPa, respectively) [8] the use of PMMA bone cements can lead to transfer of load to the material (stress shielding) and insufficient mechanical stimulation to adjacent tissue. Lack of proper mechanical loading induces bone resorption around the implant and fatigue failure of the implant [4]. In addition, a number of PMMA cements have been shown to shrink more than 5% during polymerization [7] which may lead to micromotion of the implant and an inflammatory response within the surrounding tissue.

4. Tissue Engineering

The limitations associated with the current regeneration techniques have inspired researchers to search for other methods to repair skeletal defects. Recently, new strategies to engineer bone have focused on the use of natural or synthetic materials as scaffolds for cell transplantation or conduits for bone in-growth. Various types of scaffolds have been tested, but synthetic bioresorbable polymers that degrade by hydrolysis into the body’s natural metabolites remain the most attractive. These materials are easily mass-produced, and changing the composition of the material can
precisely modulate their physical, chemical, mechanical, and degradative properties. Scaffold compositions of copolymers or polymer blends can be manufactured to create materials with properties that are advantageous over homopolymers for specific applications. Biodegradable polymers offer the possibility to completely replace osseous tissue and thus overcome problems such as infection and device dislocation associated with permanent implants [8].

4.1 Scaffold Properties

Synthetic polymeric scaffolds must possess unique properties in order to optimally enhance bone formation (see Table 1-1). The primary role of a scaffold is to provide a temporary substrate to which cells can adhere [5]. How cells attach to the scaffold can affect their behavior: strong cell adhesion and spreading are associated with proliferation, while a rounded cell shape is indicative of cell-specific function [8]. A scaffold must therefore act as a substrate to promote cell adhesion and maintenance of function without hindering proliferation. The effectiveness of a material in achieving this goal is dependent mainly on surface chemistry, which determines the interactions between cell and substrate [5]. In addition, the spatial relationship of cells within their ECM can be important for continuance of function. Therefore, three-dimensional scaffolds can assist in the organization of cell growth and the formation of ECM [5].

Porosity, pore size, and pore structure are important parameters in insuring adequate room for tissue regeneration as well as nutrient delivery to cells within a three-dimensional construct. Highly porous scaffolds with large void volume and high surface area to volume ratio are desirable to maximize space for cell seeding, attachment, growth, ECM production and vascularization. To attain the high surface area per unit volume needed for anchorage dependent cells, smaller pore diameters are preferred as long as the pore size is greater than the diameter of a cell in suspension (typically 10
\( \mu \text{m} \). However, larger pores are required for cell migration, growth, and ECM production. Previous experiments suggest that a pore diameter of 200-400 \( \mu \text{m} \) is optimal for bone ingrowth [8]. An interconnected pore network enhances diffusion within the scaffold, improving nutrient supply and waste removal and thus increasing viability of cells beyond the edges of the construct.

Initial mechanical properties of the polymer scaffold should be almost identical to the surrounding bone. If the scaffold is much stronger than bone, stress shielding occurs [4]. On a microscopic scale, the local stiffness of the polymer may affect the mechanical tension generated by a cell’s cytoskeleton, which can control cell shape and therefore function [8]. As well as providing proper support in the early stages of healing, graded load transfer is needed later in the process for creation of replacement tissue that is identical to the original. The rate of scaffold degradation, therefore, must balance the need to maintain structural support for cellular proliferation and ECM secretion with the need for polymer disintegration to leave room for new tissue in-growth. The mechanism by which the scaffold degrades and the degradation by-products should both be considered when developing a polymeric scaffold. Materials that undergo surface erosion degradation release products gradually. The continued renewal of the exterior of this material renders cell attachment and culture on these materials problematic. Materials that degrade through bulk erosion release their products only when the molecular weight of the polymer reaches a critical value. Past the critical point, mechanical properties are difficult to maintain. Additionally, when the degradation products are acidic, a burst effect may cause a substantial drop in the local pH which in turn may cause tissue necrosis or inflammation. Micromotion at the implant site or microparticles formed during the degradation process may elicit an inflammatory
response. For example, it has been demonstrated that polymer microparticles suppress initial rat marrow stromal osteoblast proliferation and differentiation in vitro [9].

Any material proposed for implantation must meet certain general criteria. The material must be biocompatible; in other words, it must not evoke an extreme adverse inflammatory or immunological response. Synthesis, processing, or sterilization techniques can affect certain factors that determine biocompatibility, including polymer chemical and physical structure and surface morphology. Residual chemicals involved in these processes may leach out of the construct and harm the surrounding native tissue and the newly formed tissue within the construct. Finally, to create a truly viable alternative to the current surgical techniques for bone repair, a polymer must be easily sterilizable, reproducibly manufacturable on a large scale, have a long shelf life, and be easy for surgeons to handle in an operating room environment.

4.2 Material Selection

Scaffold design parameters may be optimized through the selection of the proper biomaterial. As with the design parameters, the choice of the material depends greatly on its intended application. The poly(α-hydroxy acid) family which includes poly(lactic acid) (PLA), poly(glycolic acid), and poly(lactic-co-glycolic acid) copolymers (PLGA) has been extensively investigated for use as prefabricated scaffolds. These polymers are particularly attractive as they are already approved by the Food and Drug Administration for certain clinical applications. The degradation products, lactic and/or glycolic acid, are found naturally in the body and are therefore processed through normal metabolic pathways [10]. However, their build up at the implant site can lower the local pH and alter the rate of degradation.

Other polymers have been explored for use as pre-formed scaffolds for bone regeneration including poly(anhydrides) and poly(phosphazenes). Unlike poly(esters)
which degrade by bulk erosion, poly(anhydrides) degrade in a controlled fashion by surface erosion. Although this type of degradation may result in a more controlled release of by-products, these materials often display poor mechanical properties. Polyanhydride-co-imides have been developed to augment the strength of the poly(anhydride) scaffold, but imides are not biodegradable [11]. Moreover, surface eroding polymers do not facilitate cell adhesion. Poly(phosphazenes) have a phosphorous-nitrogen backbone, and are rendered hydrolytically degradable by the addition of imidazole or amino acid alkyl ester side chains. The nature of the side chain and the percent substitution can affect the degradation rate and cell attachment [10].

Poly(propylene fumarate) (PPF) is another material extensively studied for use as a tissue guidance scaffold. PPF is an unsaturated linear polyester that can be cross-linked through its fumarate double bond. PPF degrades by hydrolysis into products that occur naturally in the body [12]. In an attempt to encourage host cell attachment and migration into the scaffold, PPF has been modified with the GRGD (glycine-arginine-glycine-apartic acid) peptide sequences [13]. Both prefabricated and injectable formulations of PPF have been investigated.

Injectable polymers are valuable because they can fill irregularly shaped defects with minimal surgical intervention. In addition to the design parameters discussed above, injectable systems need to meet the additional criteria of crosslinking in situ in a timely fashion without detrimental effects to the surrounding tissue. All reagents, including the initial polymer/monomer formulations, initiators, crosslinking agents, porogens and reaction products must be non-toxic. Furthermore, reaction parameters such as heat release and pH change must be minimized.

When crosslinked in situ, PPF is injected into the defect with any one of a number of crosslinking agents (N-vinyl pyrrolidinone [14], poly(ethylene glycol)-dimethacrylate [15], or PPF-diacrylate [12] to name a few possibilities), the initiator
benzoyl peroxide, and the leachable porogen sodium chloride (NaCl). Many of the scaffold parameters can be controlled through manipulation of the method of PPF synthesis, crosslinking agent used, and ratios of initiator and crosslinking agent to PPF. Injectable formulations of PPF have been produced that reach gel point between 5-10 minutes and reach a maximum crosslinking temperature only 10°C higher than the normal physiological temperature of 37°C. In addition, they have the desired compressive strength and modulus initially and during the course of degradation for consideration for trabecular bone augmentation [14,16,17].

4.3 Scaffold Applications

One of the most promising techniques using bioresorbable polymer scaffolds for skeletal regeneration involves the transplantation of autologous cells. Pre-osteoblasts obtained from the patient can be harvested from a remote location, expanded and differentiated in culture, and seeded onto the appropriate scaffold [18]. After further culture of the cells within the scaffold, the construct is transplanted to the wound site. This method is the most widely used in tissue engineering and allows the patient's own cells to fabricate an extracellular matrix with the appropriate structural properties and signaling molecules under controlled conditions in culture. All of the prefabricated scaffold materials discussed above have been investigated for this strategy, while most of the research has focused on the use of poly(α-hydroxy acids). Osteoblast/PGA constructs implanted subcutaneously in nude mice and in full thickness rabbit calvarial defects were shown to form new bone after 20 weeks [19] and 12 weeks [20], respectively.

Sometimes fracture fixation is required immediately, leaving no time for expansion of cells in culture. In this case, a method that relies on the in-growth of bone
into the scaffold may be employed. Osteoconduction is the provision of a scaffold to enable bone growth at a time and in a location when and where it would occur because of the presence of bone forming cells and bioactive molecules from the surrounding tissue. Osteoinduction, on the other hand, is the process of effecting bone growth in a location and at a time where and when it would not occur by providing the scaffold, cells, and/or bioactive molecules [10]. Defects that require osteoinductive materials are called critical sized defects. Rat stromal osteoblasts have been shown to attach to and migrate upon three-dimensional PLGA foams in vitro [21] and in vivo [22] in non-critical sized defects, demonstrating the osteoconductivity of this material. PPF has also been shown to be osteoconductive both in vitro [23] and in vivo [24,25]. Five weeks after implantation of PPF constructs into non-critical sized rat tibial defects, the degrading scaffolds exhibited in-growth of new bone trabecuae as well as good integration with the surrounding bone [24]. The addition of peptide sequences such as GRGD [13] that modulate cellular behavior should augment PPF’s ability to assist in bone regeneration, possibly making it osteoinductive and available for use in critical sized defects.

Another technique employed to enhance bone regeneration in polymeric scaffolds is the incorporation of bioactive molecules that modulate cellular activity. The most commonly investigated osteogenic factors are the members of the transforming growth factor-β (TGF-β) superfamily which includes transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein-2 (BMP-2). One method of enhancing the in vivo efficiency of these factors is to facilitate their sustained release over an extended period time. With tissue engineered devices, there are two different potential delivery systems [26]. Growth factors can be incorporated directly into the scaffold and released by a diffusion-controlled mechanism regulated by pore morphology as the scaffold degrades. Alternately, the growth factor delivery system, in the form of microparticles,
nanoparticles, or rods can be incorporated into a scaffold. For these devices, the rate of release would depend on the degradation of the delivery device with some contribution from that of the scaffold, and rate of growth factor diffusion through the pores of the delivery system and of the scaffold. For both delivery systems, dosage, release pattern, kinetics of release, and duration of delivery are important parameters that need optimization [26].

The use of the poly(α-hydroxy acid) family of polymers has been the main area of focus for growth factor delivery devices. Unilateral critical sized defects in rabbit radii treated with a scaffold of PLA delivering rhBMP-2 demonstrated greater radiopacity as well as greater torque at failure as compared to untreated controls. [27] PLGA microparticles loaded with rhBMP-2 incorporated in an allogenic blood clot promoted bone formation in rat mandibular defects [28]. Finally, towards development of a fully synthetic carrier/scaffold system, TGF-β1 encapsulated in a blended poly(lactic-co-glycolic acid)/poly(ethylene glycol) (PLGA/PEG) microparticles was released and promoted proliferation and differentiation of osteoblast precursor cells cultured for 28 days in vitro on PPF substrates [23].

5. Conclusions

Bone performs many important functions in the human body. When bone is deficient in one location, many of these functions can be carried out by bone at distant sites, but its mechanical role in supporting the body and assisting in body movement and locomotion cannot. The mechanical functions, once lost by trauma or disease, can only be regained by restoring skeletal continuity at the location of interest.

Tissue engineering approaches that utilize polymeric, biodegradable materials hold great promise for regeneration of fully functional bone. Polymeric materials are
versatile; their material properties can be tailored to meet specific needs through alterations in polymer composition and structure. Before these techniques can become a reality for patients, however, additional work must be done. Novel biomaterials should be developed that will interact with living tissue and modulate bone formation and repair. Further investigation of the bone growth factors should be done to determine the proper release kinetics most effective for particular applications. Improvements in the carriers for these molecules may be needed to achieve the proper spatial and temporal release to enhance cellular attachment and proliferation. As more information is obtained about the biology of bone fracture and repair, current strategies will improve and new approaches will be developed, taking the use of bioresorbable polymers for the regeneration of bone closer to clinical application.
### Table 1-1: Scaffold design parameters

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porosity</td>
<td>Maximum without compromising mechanical properties</td>
</tr>
<tr>
<td>Pore Size</td>
<td>200-400 μm</td>
</tr>
<tr>
<td>Pore Structure</td>
<td>Interconnected</td>
</tr>
</tbody>
</table>

**Mechanical Properties**

| Material              | Tension & Compression: |  |
|-----------------------|------------------------|
|                       | Strength 5-10 MPa      |  |
|                       | Modulus 50-100 MPa     |  |
| Trabecular Bone       |                        |  |
|                       | Tension: Strength 80-150 MPa |  |
|                       | Modulus 17-20 GPa     |  |
|                       | Compression: Strength 130-225 MPa |  |
|                       | Modulus 17-20 MPa     |  |
|                       | Shear: Longitudinal 54-70 MPa |  |
|                       | Radial 3.3 GPa        |  |
| Cortical Bone<sup>a</sup> |                      |  |

**Degradative Properties**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degradation time&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Less than 6 months</td>
</tr>
<tr>
<td>Degradation mechanism</td>
<td>Bulk or Surface Erosion</td>
</tr>
<tr>
<td>Time to reach gel point&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Approximately 10 minutes</td>
</tr>
<tr>
<td>Biocompatibility</td>
<td>No harmful chronic inflammation</td>
</tr>
<tr>
<td>Sterilizability</td>
<td>Sterilizable without altering material properties</td>
</tr>
</tbody>
</table>

<sup>a</sup>Reported values for the strength and modulus of cortical bone were measured parallel to the long axis of the osteon.

<sup>b</sup>Degradative time in this instance refers to the time to loss of scaffold mechanical properties (time at which the scaffold no longer shares in weight bearing).

<sup>c</sup>Time to reach gel point is for injectable, *in situ* crosslinkable systems.

Table adapted from [8]
CHAPTER II

Objectives

The overall goal of this research project was to develop and characterize injectable composite materials based on poly(propylene fumarate) (PPF) for use as conduits for bone ingrowth as well as vehicles for controlled release of the 23 amino acid osteogenic peptide TP508 in bone tissue engineering applications. The engineering design goals of this system were to formulate composites that exhibit varied release kinetics of the incorporated peptide when physical properties of the composite were manipulated. The biological goals were to augment bone regeneration in segmental defects thorough assessment of varied TP508 release profiles.

The central research hypothesis was that by altering the release kinetics of an osteogenic factor, cellular response in terms of migration, differentiation, proliferation, and ECM production will be affected. We believed that, for a given osteogenic factor, an ideal dosage and release profile existed that would maximize the effect of the osteogenic factor in an osseous defect site. This work comprised of the following specific aims:

1) To develop a method for incorporating osteogenic peptides into PPF scaffolds that would allow for modulation of the peptide release kinetics.

2) To determine the influence of varying formulation parameters of the resulting PPF-based composite scaffolds on the in vitro peptide release kinetics.

3) To assess the effect of peptide incorporation method on PPF-based scaffold degradation in vitro.

4) To explore the influence of varied release kinetics from PPF-based composites on the restoration of osseous continuity in a segmental defect model.

5) To examine the effect of varied release kinetics from PPF-based composites on the in vitro degradation.
CHAPTER III

Controlled Release of an Osteogenic Peptide from Injectable Biodegradable Polymeric Composites

Abstract

Poly(DL-lactic-co-glycolic acid)/poly(ethylene glycol) (PLGA/PEG) blend microparticles loaded with the osteogenic peptide TP508 were added to a mixture of poly(propylene fumarate) (PPF), poly(propylene fumarate)-diacrylate (PPF-DA), and sodium chloride (NaCl) for the fabrication of PPF composite scaffolds that could allow for tissue ingrowth as well as for the controlled release of TP508 when implanted in an orthopedic defect site. In this study, PPF composites were fabricated and the in vitro release kinetics of TP508 were determined. TP508 loading within the PLGA/PEG microparticles, PEG content within the PLGA/PEG microparticles, the microparticle content of the PPF composite polymer component, and the leachable porogen initial mass percent of the PPF composites were varied according to a fractional factorial design and the effect of each variable on the release kinetics was determined for up to 28 days. Each composite formulation released TP508 with a unique release profile. The initial burst release (release through day 1) of the PLGA/PEG microparticles was reduced upon inclusion in the PPF composite formulations. Day 1 normalized cumulative mass release from PPF composites ranged from $0.14 \pm 0.01$ to $0.41 \pm 0.01$, whereas the release from PLGA/PEG microparticles ranged from $0.31 \pm 0.02$ to $0.58 \pm 0.01$. After 28 days, PPF composites released $53 \pm 4\%$ to $86 \pm 2\%$ of the entrapped peptide resulting in cumulative mass releases ranging from $0.14 \pm 0.01 \mu g$ TP508/mm$^3$

*This chapter was published as: E.L. Hedberg, A. Tang, R.S. Crowther, D.H. Carney, A.G. Mikos, "Controlled release of an osteogenic peptide from biodegradable polymeric composites," J Control Rel, 2002; vol. 89, p. 137-150.
scaffold to $2.46 \pm 0.05 \mu g$ TP508/mm$^3$ scaffold. The results presented demonstrate that alterations in the composite's composition can lead to modulation of the TP508 release kinetics. These composites can be used to explore the effects varied release kinetics and dosages on the formation of bone in vivo.

1. Introduction

Current therapies for the repair of osseous defects often utilize grafted tissue either from the patient (autograft) or from cadavers (allograft). Autografts are preferred as they allow for the transplantation of the patient's own living tissue including its vascularization and resident cell population [1]. Unfortunately, the amount of autograft tissue available for transplantation is greatly limited. Allografts are usually processed prior to implantation to avoid rejection. Because of the processing, these grafts may not possess viable cells but may retain the growth factors and other bioactive molecules contained within the extracellular matrix. These grafts are successful as they may serve both as scaffolds for cellular ingrowth and as a source for healing factors that promote bone ingrowth. However, disease transmission is a concern associated with the use of allografts [2]. Since neither the autograft nor the allografts provide the patient with ideal bone graft material, there is a strong clinical need to develop alternatives to these methods. Recent advances in tissue engineering have focused on the use of biodegradable polymers as scaffolds for guided cellular ingrowth, cell transplantation, or the delivery of therapeutic molecules as methods for regenerating osseous tissue.

Since Urist et al. demonstrated that glycoproteins extracted from demineralized rabbit tibia matrix could induce bone formation in ectopic sites in rabbits and mice, tremendous advances have been made in the development of recombinant growth factors, proteins, and peptides for bone tissue regeneration [3]. The most widely
explored factors are members of the transforming growth factor β (TGF-β) super-family including transforming growth factor β1 (TGF-β1), bone morphogenetic protein 2 (BMP-2), and osteogenic protein (OP-1 or BMP-7) [4]. BMP’s have been shown to induce bone formation within a defect without the use of a carrier, but their relatively short-half lives necessitate the use of significant amounts of protein [5]. To increase the \textit{in vivo} efficacy as well as reduce the quantities needed, the development of polymeric carriers capable of controlled, sustained delivery of proteins and peptides is desirable.

Several natural and synthetic polymers have been explored for use as delivery vehicles for bone inductive molecules. The poly(α-hydroxy acid) family of polymers including poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymers poly(DL-lactic-co-glycolic acid) (PLGA), have been the focus of much of this research because they are biocompatible, undergo controllable hydrolytic degradation into natural metabolites, and can be processed into many forms [6-8]. PLGA microparticles have been used to release TGF-β1 and BMP-2 [9-13]. The rate of release of bioactive molecules can be modulated by altering the molecular weight of the PLGA as well as the ratio of lactic to glycolic acid in the copolymer [7]. The addition of the much more hydrophilic polymer poly(ethylene glycol) (PEG) to form PLGA/PEG blend microparticle carriers allows for further attenuation of the loaded compounds [14].

The augmentation of bone formation with the release of growth factors via PLGA microparticle carriers has confirmed the need for controlled release vehicles for successful tissue regeneration. Microparticles alone are inherently limited, however, as they do not offer a substrate for cellular migration and proliferation. To overcome this obstacle, highly porous three-dimensional scaffolds have begun to be explored. Sustained release of rhBMP-2 has been achieved through the adsorption of the protein onto the surface of the PLGA after fabrication of the porous foams [15]. Controlled
release of therapeutic agents has also been achieved through their incorporation into PLGA matrices through a number of different fabrication techniques [16-19]. The rate of release of bioactive molecules from PLGA scaffolds has been shown to be predominately diffusion controlled and modulated by the pore size and protein loading of the matrices [18].

In order to minimize the surgical intervention required for the implantation of the controlled release scaffolds, the development of materials that can be injected and cross-linked in situ would be desirable. One potential candidate for this application is the synthetic polymer poly(propylene fumarate) (PPF). PPF is a linear polyester that can be cross-linked through its many unsaturated double bonds. The cross-linked polymer degrades by hydrolysis into products that occur naturally in the body [20]. When cross-linked in situ, PPF is injected into the defect with any one of a number of cross-linking agents (N-vinyl pyrrolidone [21], poly(ethylene glycol)-dimethacrylate [22], or PPF-diacylate [20] to name a few possibilities), the free-radical initiator benzoyl peroxide (BP), and the leachable porogen sodium chloride (NaCl) and cross-linked via free radical propagation. Many of the scaffold parameters can be controlled through manipulation of the method of PPF synthesis, cross-linking agent used, and ratios of initiator and cross-linking agent to PPF. Injectable formulations of PPF have been produced that reach gel point between 5-10 minutes and reach a maximum cross-linking temperature only 10°C higher than the normal physiological temperature of 37°C. In addition, PPF scaffolds possess the desired compressive strength and modulus both initially and during the course of degradation for consideration for trabecular bone augmentation [21,23,24]. PPF substrates have been shown to be osteoconductive both in vitro [25,26] and in vivo [27-29].
PPF is currently being explored in our laboratory as a scaffold material for the controlled, localized delivery of osteoinductive proteins and peptides. This study focuses on the release of TP508 (Chrysalin®) from PPF composite scaffolds. TP508 is a 23 amino acid synthetic peptide representing the non-proteolytic receptor-binding domain of thrombin [30,31]. TP508’s action in bone regeneration has not yet been fully elucidated, but previous research has shown that the release of TP508 from PLGA microparticles results in augmented bone regeneration compared to untreated controls in rabbit ulnar segmental defects [32]. During normal tissue repair, thrombin becomes sequestered within newly forming fibrin clots and is stored there until the clots degrade [33]. Because thrombin release from a defect is naturally sustained, prolonged release of TP508 should offer the greatest efficacy in tissue engineering applications.

In this study, TP508-loaded PLGA/PEG microparticles were combined with PPF, PPF-DA, and the leachable porogen sodium chloride (NaCl) to form PPF composites for the controlled release of TP508. We determined: 1) whether PPF composites could be fabricated with varied release kinetics of TP508 and, 2) whether the systematic alteration of the composite’s microstructure could lead to modulation of the release kinetics of the entrapped TP508. To address these questions, four variables within the composite formulation were examined in a Resolution III two level fractional factorial design. These variables were: 1) the TP508 loading within the PLGA microparticles, 2) the inclusion of the hydrophilic co-factor PEG within the PLGA microparticles, 3) the microparticle content of the PPF composite polymer component, and 4) the leachable porogen initial mass percent of the PPF composites. The effects of these parameters on the release kinetics of TP508 were evaluated.
2. Materials and Methods

2.1 Materials

2.1.1 PPF and PPF-DA Synthesis

Diethyl fumarate, 1,2 propanediol (propylene glycol), and anhydrous potassium carbonate were purchased from Acros Chemical (Pittsburgh, PA) and used as received. Solvents for PPF purification were supplied by Fisher Scientific (Pittsburgh, PA).

Acryloyl chloride, 2-butanol, fumaric acid, pyridine, and triethylamine were purchased from Aldrich Chemical (Milwaukee, WI). Propylene oxide was purchased from Acros Chemical. Magnesium sulfate, sodium sulfate, and all remaining organic solvents were purchased from Fisher Scientific. All polymer synthesis chemicals were used as received.

2.1.2 Microparticle Fabrication

PLGA with a 50:50 lactic to glycolic acid copolymer ratio (Medisorb®, Alkermes, Cincinnati, OH) was used in this study. Gel permeation chromatography was used to determine the weight average molecular weight ($M_w$) of the PLGA (47,600 ± 500) and the polydispersity index (PI, 1.73 ± 0.05; n = 4). The PI is equal to the ratio of the weight average molecular weight to number average molecular weight ($M_w/M_n$). Poly(ethylene glycol) (PEG, $M_w$=10,700) and poly(vinyl alcohol) (PVA, 88 mol% hydrolyzed, $M_w$=13,000-23,000) were purchased from Aldrich Chemical. Dry, unlabeled TP508 and an I$^{125}$ labeled TP508 solution (286 μg/mL) was provided by Chrysalis Biotechnology, Inc. (Galveston, TX).
2.1.3 Scaffold Fabrication and Release Kinetics Study

Benzoyl peroxide (BP) and N,N-dimethyl-p-toluidine (DMT) were purchased from Aldrich Chemical and used as received. Sodium chloride (Fisher Scientific) was sieved to a particle diameter of 300 - 500 μm.

2.2 Methods

2.2.1 PPF Synthesis

PPF was synthesized following a two-stage procedure [34]. Diethyl fumarate and propylene glycol were added to a 1L three-necked round bottom flask in a molar ratio of 1:3, respectively. In addition, zinc chloride was added as a catalyst in a 0.01:1 molar ratio to diethyl fumarate, and methylene chloride added for use as a solvent. The reaction mixture was maintained under nitrogen and mechanically mixed using an overhead stirrer. The flask was submerged in an oil bath set at a temperature of 100°C. The oil bath temperature was gradually raised to 150°C in 10°C increments over the course of one hour. Ethanol, a byproduct of the reaction, was collected as a distillate. The reaction was run for 8 h to produce the diester intermediate bis(2-hydroxypropyl) fumarate.

The reaction mixture was allowed to cool to 100°C and then placed under vacuum (<1 mmHg). The temperature was increased in 10°C increments every 15 min until 150°C was reached. During the transesterification of the intermediate, propylene glycol was driven off and collected as a distillate. The reaction was run for 10 h after the application of the vacuum.

For purification, a 5% w/w solution of 1 N HCl was mixed with the reaction solution in a separatory funnel to remove the zinc chloride. The product was washed in
a similar manner with two washes each of ddH2O and a saturated NaCl solution. To remove residual water, the polymeric solution was mixed with an excess amount of anhydrous sodium sulfate. The drying agent was then removed by vacuum filtration. The product was concentrated by rotary evaporation and dried under vacuum (<1 mmHg, 12 h). The final purified PPF was a clear viscous liquid of number average molecular weight (Mn) 2,180 ± 20 and polydispersity index (PI) 1.74 ± 0.01, as determined by gel permeation chromatography compared to polystyrene standards.

2.2.2 PPF-DA Synthesis

Fumaric acid (1 mol) and pyridine (0.03 mol) were added to a 1L three-necked round bottom flask containing the solvent 2-butane. The flask was submerged in an oil bath set at 85°C. The reaction mixture was maintained under nitrogen and mechanically mixed using an overhead stirrer. A condenser and drying tube were added to one neck of the flask to allow for the reflux of unreacted propylene oxide. Propylene oxide (3 mol) was added drop-wise over the course of four hours and the reaction mixture was left to stir overnight. Since fumaric acid is insoluble in 2-butane, the reaction was considered complete upon total dissolution of the white fumaric acid precipitate. Upon completion of the reaction, propylene oxide was removed through distillation at atmospheric pressure. After removal of propylene oxide, the system pressure was decreased with aspiration to allow for distillation of the 2-butane. For purification, a 5% w/w solution of 1 N NaOH was mixed with the reaction solution in a separatory funnel. The product was then washed in a similar manner with ddH2O and a saturated NaCl solution. The product, bis(hydroxypropyl) fumarate, was mixed with an excess of anhydrous magnesium sulfate to remove residual water. The drying agent
was removed via vacuum filtration. The resulting material was condensed via rotary evaporation and dried under vacuum (<1 mmHg) for 12 h.

To synthesize PPF-DA, acryloyl chloride (3 mol) was added drop-wise over the course of 12 h to 1L three-necked round bottom flask containing bis(hydroxypropyl) fumarate (1 mol), triethyleamine (3 mol), and anhydrous methylene chloride. The reaction mixture was maintained at a temperature of −5°C with a cooling bath during the addition of the acryloyl chloride. After the addition was complete, the cooling bath was removed and the mixture was allowed to warm to room temperature and left to stir overnight. The mixture was maintained under nitrogen and stirred with a magnetic stir bar throughout the reaction.

For purification, methylene chloride was removed by rotary evaporation. To extract PPF-DA from the remaining solution, ethyl ether was mixed with the reaction mixture and then decanted. PPF-DA is soluble in ethyl ether while the triethyleamine salt byproducts are not. After five serial washings of the reaction mixture, the ethyl ether solutions were combined and the PPF-DA was condensed through rotary evaporation. For further purification, the PPF-DA was dissolved in methylene chloride and washed with a 5% w/w solution of 1 N NaOH, ddH₂O and a saturated NaCl solution as described above. Residual water was extracted through the addition of excess anhydrous magnesium sulfate which was then removed by vacuum filtration. Methylene chloride was removed through rotary evaporation and vacuum drying (<1 mmHg, 12 h). Finally, the PPF-DA was resuspended in a mixture of ethyl ether and petroleum ether (1:2 by volume) and eluted through a 7-inch silica gel column. The purified PPF-DA was condensed and dried through rotary evaporation and vacuum drying, respectively.
2.2.3 TP508 Solution Preparation

The peptide solutions used for entrapment of TP508 into PLGA microparticles were obtained by adding a trace amount of $^{125}$-TP508 to unlabeled peptide solutions in the “hot” ($^{125}$-TP508) to “cold” (TP508) mass ratio of 1: 1.6 x $10^3$. For microparticles loaded with 0.15 g TP508/ g microparticle, solutions of 0.3 mg TP508/ µL ddH$_2$O were utilized. Solutions of 0.1 mg TP508/ µL ddH$_2$O were made for fabrication of microparticles with 0.05 g TP508/ g microparticle.

2.2.4 Microparticle Fabrication

Microparticles of 50:50 PLGA and PEG loaded with TP508 were prepared by a double-emulsion, solvent extraction technique ((water-in-oil)-in water) as previously described [14,35]. Briefly, 125 µl of a given TP508 solution was injected into a flint glass tube containing the appropriate amounts of PLGA and PEG in 1 mL dichloromethane. This mixture was emulsified on a vortexer (Vortex Genie 2, Scientific Industries, Bohemia, NY). The first emulsion was then combined with 1.5 mL 0.3% aqueous PVA solution with vortexing, producing a second emulsion. This second emulsion was added to 100 mL of 0.2% aqueous isopropanol and 98.5 mL of 0.3% aqueous PVA with rapid stirring for 1 h. Microparticles were formed through the extraction of the organic solvent dichloromethane into the aqueous phase, resulting in the precipitation of the dissolved polymer and subsequent microparticle formation. The microparticle suspension was centrifuged at 180 x g for 1 min. The microparticles were lyophilized to dryness and the mass of microparticles formed was recorded.
2.2.5 Microparticle Characterization

The mass of TP508 per batch of microparticles (initial mg TP508/batch) represents the initial mass of TP508 added to the microparticle fabrication process. Dividing the mass of TP508 per batch of microparticles (initial mg TP508/batch) by the mass of microparticles fabricated per batch (mg microparticles/batch) yields the theoretical loading (mg TP508/mg microparticles). Actual loading of TP508 within the microparticles was determined by placing approximately 5 mg of microparticles in 5 mL polyethylene vials and measuring the γ-emmitance of the $^{125}$-TP508 trace molecules (Cobra II Autogamma, Packard, Meridian, CT). The mass of TP508 released was obtained through comparison of the measured activity with a calibration curve constructed through serial dilutions of the $^{125}$-TP508 stock solutions. Entrapment efficiency was calculated by dividing the actual loading by the theoretical loading and multiplying by 100%.

2.2.6 Microparticle Experimental Design

A study to assess the TP508 release kinetics from PLGA/PEG microparticles was designed based on a two-level full factorial design varying two parameters[36]: (1) TP508 loading in the microparticles and (2) PEG content of the microparticles. The high and low values for each parameter are presented in Table 3-1(a) and the formulation combinations are presented in Table 3-1(b). The effects of the parameters on the TP508 release kinetics over 28 days were evaluated. The main effects of each parameter were determined for normalized cumulative release for days 1, 4, 7, and 28.
2.2.7 Preparation of Porous PPF/PPF-DA Based Composites

In a typical procedure for the preparation of composites using PPF-DA as a crosslinker, 1.0 g PPF was mixed with 2.05 g PPF-DA (the double bond ratio of PPF/PPF-DA was 0.5) in the presence of CH₂Cl₂ and stirred overnight at room temperature. After removal of the solvent, 0.14 mL of initiator solution (0.1 g BP per 1 mL diethyl fumarate) was added to the PPF/PPF-DA mixture and agitated thoroughly. Proper amounts of microparticles and NaCl were each added to the mixture and agitated until they appeared to be homogeneously distributed. Finally, 5 μL of DMT was injected into the mixture with vigorous mixing. The material was inserted into glass cylindrical vials (diameter = 6.5 mm) and placed in a 37°C water bath for 20 min. The crosslinked samples were removed from the molds and cut to a length of 13 mm.

2.2.8 PPF Composite Experimental Design

PPF composite formulations consisting of PPF, PPF-DA (crosslinking molecule), TP508 loaded PLGA/PEG microparticles (peptide microparticle carrier), and NaCl (leachable porogen) were fabricated according to a Resolution III two-level fractional design [36]. In this study, four variables were considered: 1) TP508 loading within the PLGA microparticles, 2) PEG content within the PLGA microparticles, 3) the microparticle content in the composites, and 4) the NaCl initial mass percent of the composites. High and low values were chosen for each parameter, and these levels were combined according to the fractional factorial design to create eight composite formulations. The values for all parameters and combinations for all formulations are presented in Table 3-2(a) and Table 3-2(b), respectively. The cumulative TP508 mass fractions and cumulative TP508 masses released were evaluated over 28 days. The main effect of each parameter was determined for days 1, 4, 14, and 28. The factorial
design demonstrated the effect each parameter exhibits while minimizing the number of trials.

2.2.9 Release Kinetics of I-125 labeled TP508 from Microparticles

Samples were maintained in glass vials containing 4 mL of pH 7.4 phosphate buffer saline (PBS) at 37°C with agitation (~70 rpm) for various time-periods up to 28 days. At the end of 0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16, 19, 22, 25, and 28 days, solution samples were collected and stored at −20°C until analysis. In order to minimize microparticle loss during sampling, microparticle samples were centrifuged for approximately 1 min at 180 x g prior to solution sample removal. Samples were resuspended in fresh PBS and returned to 37°C with agitation. Tracer I\textsuperscript{125}-TP508 activity of collected solution samples was measured on a γ-counter. TP508 mass released was determined as described above.

Statistical Analysis

All data are reported as means ± SD for n = 3. An Analysis of Variance (ANOVA) and Tukey's multiple comparison tests were employed at significance levels of 95%.

3. Results

3.1 Microparticle Entrapment Efficiency

PLGA/PEG blend microparticles were fabricated using the double-emulsion, solvent-extraction technique previously described [14,35]. The entrapment efficiencies of TP508 in the microparticles are presented in Table 3-3. The entrapment efficiencies of TP508 were statistically different for all formulations examined (p < 0.05), indicating that both PEG content and TP508 loading influenced the entrapment efficiency.
Microparticles with high (15 wt%) TP508 loading demonstrated lower entrapment efficiencies (38 ± 1 and 46 ± 2 for 5 and 0 wt% PEG, respectively) than those of microparticles loaded with low (5 wt%) TP508 loading (62 ± 3 and 81 ± 4). Formulations of 0 wt% PEG exhibited statistically greater entrapment efficiencies than those fabricated with 5 wt% PEG. This difference may be due to leaching of the TP508 with soluble PEG into the external aqueous phase during microparticle fabrication.

3.2 In Vitro TP508 Release Kinetics from PLGA/PEG Microparticles

Release kinetics of TP508 from PLGA/PEG microparticles was monitored over a 28-day period (Figure 3-1(a)). Microparticle formulations prepared with 15 wt% TP508 displayed initial release by day 1 of 0.51 ± 0.01 μg TP508 released/μg TP508 entrapped (MP1) and 0.44 ± 0.01 μg TP508 released/μg TP508 entrapped (MP2), while microparticles prepared with 5 wt% TP508 showed smaller burst releases of 0.29 ± 0.01 μg TP508 released/μg TP508 entrapped (MP3) and 0.23 ± 0.02 μg TP508 released/μg TP508 entrapped (MP4) (Figure 3-1(b)). For both high and low initial TP508 loadings, microparticle formulations with 5 wt% PEG had greater percentage of entrapped TP508 released during the first 24 h than those with an initial PEG content of 0 wt%. As release continued, the effect of increasing TP508 initial loading from 5 to 15-wt% on normalized cumulative mass release decreased (Figure 3-2). The effect of PEG content remained relatively constant over days 1, 4, and 14. By day 28 however, an increase in PEG content from 0 to 5 wt% caused an increase in the amount of TP508 released from the PLGA/PEG microparticles.

3.3 In Vitro TP508 Release Kinetics from PPF Composites
A $2^{4-1}$ fractional factorial design was conducted to assess the effects of microparticle TP508 loading, microparticle PEG content, scaffold microparticle loading, and scaffold initial NaCl content on the in vitro release of TP508 from PPF composites impregnated with peptide loaded PLGA/PEG microparticles. The eight formulations examined displayed release profiles that varied in their extent of initial burst, rate of release, and total percentage of entrapped TP508 released after 28 days in vitro (Figure 3-3). The four parameters studied differed in their effect on normalized cumulative release. Increasing either the TP508 loading or PEG content of the PLGA/PEG microparticles led to an increase in the percentage of TP508 mass released for all days considered (Figure 3-4). Changing microparticle loading from 0.03 to 0.09-wt% of the composite polymer mass caused a decrease in the normalized cumulative release on days 1 and 14, while affecting an increase by day 28. On days 1 and 4, an increase in the initial composite sodium chloride content from 75 to 80-wt% brought about an increase in the mass percentage of TP508 released. An increase in this parameter at the later time points led to a decrease in normalized cumulative mass released.

Figure 3-5 presents the normalized cumulative TP508 mass released after 24 h from PLGA/PEG microparticles and the corresponding PPF composites. For all formulations, embedding of the microparticles in the PPF composites resulted in a statistically significant reduction ($p < 0.01$) of the initial burst of TP508 released.

TP508 total mass release kinetics for all formulations is presented in Figure 3-6. Day 28 cumulative dosages varied from $0.14 \pm 0.01 \mu g/mm^3$ scaffold (F8) to $2.46 \pm 0.05 \mu g/mm^3$ scaffold (F1). Increasing the loading of TP508 and PEG within the embedded microparticles and the microparticle content of the composites from their low to high values (see Table 3-2(a)) all led to an increase in the cumulative mass released on all days examined (Figure 3-7). Increasing the initial sodium chloride weight fraction had no
effect on days 1 and 4, and resulted in a decrease in the percentage of TP508 released on days 14 and 28.

4. Discussion

To promote bone regeneration via localized delivery of bioactive molecules, it will be necessary to develop materials that can act both as a scaffold for the support of cellular migration, proliferation, and differentiation, as well as a delivery vehicle for the controlled release of osteoinductive proteins and peptides. To address this need, PPF composite materials consisting of PPF, the cross-linking agent PPF-DA, TP508 loaded PLGA/PEG microparticles, and a leachable porogen (NaCl) were constructed. These PPF composite materials were thermally cross-linked after the addition of BP and DMT to form PPF composites. In the present study, the release kinetics of TP508 from PPF composites were investigated to determine whether PPF composites could be used for the controlled release of TP508 and, if so, whether systematic manipulation of the composite's microstructure would result in alterations of the TP508 release kinetics.

PPF composite formulations were fabricated according to a Resolution III two level fractional factorial design with high and low values of the TP508 loading within the PLGA microparticles, PEG content within the PLGA microparticles, microparticle loading of the PPF composite polymer component, and leachable porogen initial mass percent of the PPF composites. This design resulted in eight different formulations. Over the course of 28 days, PPF composites were maintained in PBS at 37°C with agitation and the in vitro release of TP508 was determined. Each PPF composite formulation examined released TP508 with a unique release profile with variations in release parameters such as the extent of burst release, the rate of release of the peptide following the initial burst, the mass fraction of TP508 released after 28 days, and the total mass of TP508 released after 28 days. Using a fractional factorial design, the
effect of increasing each variable from its low to high value was determined [36]. On the
days examined (days 1, 4, 24, and 28), an alteration in the TP508 and PEG content of
the microparticles, microparticle loading of the composite polymer component, or initial
NaCl weight percent resulted in a change in the cumulative mass percent and
cumulative mass of TP508 released.

To load TP508 into the PPF composites, PLGA microparticles were utilized. Previous work has shown that TP508 can be incorporated in PLGA microparticles and released while maintaining its bioactivity [32]. The incorporation of the peptide into microparticle carriers allowed for the distribution of the hydrophilic peptide in a hydrophobic polymer scaffold as well as shielding of the peptide from any adverse effects of exposure to the free radical polymerization during cross-linking of the composite. PLGA is not soluble in any of the liquid components of the composite formulations (PPF, PPF-DA, and diethyl fumarate). The addition of PLGA microparticles to a mixture of these components, therefore, is not expected to adversely affect the integrity of the PLGA microparticles.

The utilization of PLGA/PEG microparticle carriers also allows for greater flexibility of the composite material, offering additional variables for the fine-tuning of the TP508 release kinetics. As seen in Figure 3-3, the composition of the microparticles influences the release of TP508 from the composites. There are a number of microparticle parameters that affect the release kinetics of a protein or peptide from PLGA microparticles such as the inclusion of co-encapsulation molecules, the use of PLGA with varying molecular weights and/or lactic to glycolic acid ratios, and the utilization of different microparticle fabrication methods [7,8,14]. These parameters were not included in this study, but it is likely that modifications of these properties could lead to further alterations in the release kinetics of TP508 from PPF composites.
Although PLGA microparticles offer many parameters that can be adjusted for the attenuation of the release kinetics, PLGA microparticles often exhibit an initial burst release of the readily solubilized peptides on the surface of the particle [14]. In this study, the embedding of the microparticles in the polymer component of the PPF composites was shown to significantly reduce the extent of the burst effect. As the release kinetics were determined using radioactively-labeled peptides, no conclusions can be made from these data regarding possible structural changes such as acylation of the peptide [37] due to its incorporation in and release from PPF composites.

Release of a peptide from hydrolytically degradable materials such as PLGA, PPF, and PPF-DA will be dependent on diffusion of the peptide through the polymer matrix. Once the sodium chloride porogen has been leached from the composites, a porous polymer scaffold remains. Initial TP508 release from the composites will be a result of the readily solubilized peptide located at the surfaces of the scaffold in contact with the aqueous environment. Contact with the aqueous environment will also allow for the degradation of the polymer components. Degradation of the readily accessible PLGA microparticles will reduce the resistance of the PLGA’s resistances to diffusion, allowing for the release of more peptide. Finally, the PPF/PPF-DA network will begin to degrade, exposing the formally sequestered microparticles.

Work by Fisher et al. has demonstrated that increasing the initial NaCl content of PPF based materials increases the porosity of the resulting polymer scaffolds [38]. The use of similarly sized NaCl particles (300 – 500 µm) to increase the porosity in different composite formulations will lead to scaffolds with increased surface areas. With an increase in surface area, the number of microparticles exposed will increase, resulting in an increase in the normalized cumulative mass released on days 2 and 7, as demonstrated in Figure 3-7. Release on day 14 and 28, however, was shown to decrease with the increase in initial porogen content. This retardation in the release of
TP508 may be attributed to the adsorption of the peptide onto the PPF/PPF-DA surfaces within the scaffold. Retardation of protein release from PPF scaffolds has been demonstrated in a study conducted by Vehof et al. [39]. TGF-β1 was incorporated into PPF scaffolds through the addition of aqueous solutions of TGF-β1 to the surface of the scaffolds. Release of TGF-β1 from these scaffolds was maintained over the course of 28 days, indicating the release of the protein was not solely based on diffusion of the protein through an aqueous media.

In order for a controlled release vehicle to be successful in orthopedic tissue engineering, it needs to possess certain engineering or structural properties in addition to the biological ones imparted by the release of a bioactive peptide [40]. The material must be biocompatible and biodegradable, and the degradation products must be readily processed or eliminated by the body. The material needs to possess initial mechanical properties to support the surrounding tissue, and the degradation rate of the material must permit the transfer of the mechanical properties from the implant material to newly formed bone. To allow for room for bone ingrowth, the material should be able to be fabricated into highly porous scaffolds with a high surface area to volume ratio. The surface of the material should support marrow stromal cell attachment, migration, proliferation, and differentiation [1].

A number of materials currently under investigation offer many of these properties, including PLGA and PPF. As an injectable formulation, however, PPF offers a number of additional attributes. The components of the PPF composite materials can be pre-fabricated and then stored to create and "off the shelf" product for clinical applications. The components can be chosen and mixed in the surgical suite within minutes, allowing the surgeon to determine the desired formulation in accordance with each patient's needs. The composite material can then be injected directly into
irregularly shaped defects and cured in situ with minimal surgical intervention. The versatility and ease of handling of PPF composite materials make these materials potentially highly attractive to the orthopedic community.

PPF composite materials also offer the research community a tool for the assessment of bone tissue regeneration in response to the release of inductive molecules. Although great strides have been made in the development and application of controlled release vehicles, it is still unclear the desired release profiles and dosages for individual osteoinductive molecules for optimal bone regeneration. Manipulation of PPF composite's microscopic structure leads to a variety of release profiles with minimal effect on the macroscopic structure of the resulting polymeric scaffold. This material, therefore, can be employed for the assessment of varied release kinetics on bone tissue response. In addition, using a homogeneous mixture of unloaded and peptide loaded PLGA microparticles, tissue response to different dosages with the same release kinetics can be examined.

5. Conclusions

Osteoinductive scaffolds for bone tissue engineering have been created through the addition of TP508 loaded PLGA/PEG microparticles into the polymeric phase of PPF composite materials. This study was conducted to determine whether TP508 could be released from PPF composites and whether the release of TP508 could be controlled though systematic manipulation of the composite's composition. By varying TP508 loading, PEG content, microparticle loading, and initial porogen weight percent, a wide variety of release profiles were obtained and the influence of each factor was determined. This material holds great promise for clinical use as well as fundamental research. Many studies investigating the use of proteins for bone regeneration use protein masses much greater than those found physiologically perhaps due to temporal
requirements not yet met by the current vehicles available for protein delivery. By varying protein release profiles from PPF composites systematically, appropriate release kinetics and dosages can be ascertained and ultimately implemented clinically.
Table 3-1: Microparticle Experimental Design

(a) High and Low Levels for Parameters Tested in the $2^2$ Design

<table>
<thead>
<tr>
<th></th>
<th>g TP508/ g Microparticle*</th>
<th>g PEG/ g Microparticle*</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Level (+)</td>
<td>0.15</td>
<td>0.05</td>
</tr>
<tr>
<td>Low Level (-)</td>
<td>0.05</td>
<td>0</td>
</tr>
</tbody>
</table>

(b) Combinations of the Experimental Values in the $2^2$ Design

<table>
<thead>
<tr>
<th>Formulation</th>
<th>g TP508/ g Microparticle*</th>
<th>g PEG/ g Microparticle*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MP2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MP3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MP4</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Microparticle comprised of PLGA, PEG, and TP508
Table 3-2: PPF Composite Experimental Design

(a) High and Low Levels for Parameters Tested in the Resolution III, Two-Level Fractional Factorial Design

<table>
<thead>
<tr>
<th></th>
<th>g TP508/ g Microparticle*</th>
<th>g PEG/ g Microparticle*</th>
<th>g Microparticle/ g Composite Polymer**</th>
<th>g NaCl / g Composite#</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Level (+)</td>
<td>0.15</td>
<td>0.05</td>
<td>0.09</td>
<td>0.85</td>
</tr>
<tr>
<td>Low Level (-)</td>
<td>0.05</td>
<td>0.00</td>
<td>0.03</td>
<td>0.70</td>
</tr>
</tbody>
</table>

(b) Combinations of the Experimental Values in the Resolution III, Two-Level Fractional Factorial Design

<table>
<thead>
<tr>
<th>Formulation</th>
<th>g TP508/ g Microparticle*</th>
<th>g PEG/ g Microparticle*</th>
<th>g Microparticle/ g Composite Polymer**</th>
<th>g NaCl / g Composite#</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>F2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>F3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F4</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F5</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F6</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F7</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>F8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* Microparticle comprised of PLGA, PEG, and TP508
** Composite polymer comprised of PPF, PPF-DA, and microparticles
# Composite comprised of PPF, PPF-DA, microparticles, and NaCl
### Table 3-3: Entrapment Efficiencies of TP508 in PLGA/PEG Microparticles

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Initial $\mu$g TP508/ Batch</th>
<th>mg Microparticle/ Batch</th>
<th>Theoretical Loading, $\mu$g TP508/ mg Microparticle</th>
<th>Actual Loading, $\mu$g TP508/ mg Microparticle</th>
<th>TP508 Entrapment Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP1</td>
<td>$37.5 \times 10^3$</td>
<td>190 ± 6</td>
<td>197 ± 6</td>
<td>75 ± 1</td>
<td>38 ± 1</td>
</tr>
<tr>
<td>MP2</td>
<td>$37.5 \times 10^3$</td>
<td>183 ± 5</td>
<td>204 ± 6</td>
<td>94 ± 2</td>
<td>46 ± 2</td>
</tr>
<tr>
<td>MP3</td>
<td>$12.5 \times 10^3$</td>
<td>208 ± 8</td>
<td>60 ± 2</td>
<td>38 ± 1</td>
<td>62 ± 3</td>
</tr>
<tr>
<td>MP4</td>
<td>$12.5 \times 10^3$</td>
<td>211 ± 6</td>
<td>59 ± 2</td>
<td>48 ± 2</td>
<td>81 ± 4</td>
</tr>
</tbody>
</table>
Figure 3-1 Cumulative release kinetics of TP508 from PLGA/PEG microparticles into PBS at 37°C with agitation (70 rpm) expressed as normalized mass release: (A) The cumulative normalized mass release for all time points; (B) the cumulative normalized mass released on days 1, 4, 14, and 28; error bars represent means±S.D. for n=3.
Figure 3-2: Main effects of TP508 loading and PEG content on normalized cumulative mass of TP508 released from PLGA/PEG microparticles for days 1, 4, 14, 28. A positive number indicates that the particular parameter had an increasing effect on the normalized cumulative mass released as the value was changed from a low (L) level (-) to a high (H) level (+) [see Table 3-1 (a)]. Error bars represent the standard errors of the effect (day 1, 0.01; day 4, 0.03; day 14, 0.01; day 28, 0.02).
Figure 3-3: Cumulative release kinetics of TP508 from PPF composite scaffolds into PBS at 37°C with agitation (70 rpm): (A) the cumulative normalized mass released for all time points; error bars are excluded for clarity. (B) the cumulative normalized mass released on days 1, 4, 14, and 28; error bars represent means±S.D. for n=3.
Figure 3-4: Main effects of microparticle TP508 loading, microparticle PE content, scaffold polymer microparticle loading, and scaffold weight percent of NaCl on normalized cumulative mass of TP508 release from PPF composite scaffolds for days 1, 4, 14, and 28. A positive number indicates that the particular parameter had an increasing effect on the normalized cumulative mass released as the value was changed from a low (L) level (-) to a high (H) level (+) [see Table 3-1 (a)]. A negative number indicates a decrease in the normalized cumulative mass release as the parameter was changed from low (L) level (-) to a high (H) level (+). Error bars represent the standard errors of the effect (day 1, 0.006; day 4, 0.007; day 14, 0.01; day 28, 0.009).
Figure 3-5: Day 1 normalized cumulative mass of TP508 released from PLGA/PEG microparticles and the PPF composites into which the PLGA/PEG microparticles were embedded. PPF composite formulations exhibited statistically lower normalized cumulative TP508 mass release (P<0.05) than the corresponding non-embedded microparticles. Error bars represent means±S.D. for n=3.
Figure 3-6: Cumulative release kinetics of TP508 from PPF composite scaffolds into PBS at 37°C with agitation (70 rpm) expressed as cumulative mass released per scaffold volume: (A) the cumulative mass released for all time points, error bars are excluded for clarity; (B) the cumulative mass released on days 1, 4, 14, and 28; error bars represent means±S.D. for n=3.
Figure 3-7: Main effects of microparticle TP508 loading, microparticle PEG content, scaffold polymer microparticle loading, and scaffold weight percent of NaCl on cumulative mass of TP508 released from PPF composite scaffolds for days 1, 4, 14, and 28. A positive number indicates that the particular parameter had an increasing effect on the normalized cumulative mass released as the value was changed from a low (L) level (-) to a high (H) level (+) [see Table 1(a)]. A negative number indicates a decrease in the normalized cumulative mass released as the parameter was changed from the low (L) level (-) to a high (H) level (+). Error bars represent the standard errors of the effect (day 1, 0.009; day 4, 0.009; day 14, 0.01; day 28, 0.01).
CHAPTER IV  

In Vitro Degradation of Porous Poly(Propylene Fumarate)/Poly(DL-Lactic-co-Glycolic Acid) Composite Scaffolds

Abstract

This study investigated the in vitro degradation of porous poly(propylene fumarate) (PPF) based composites incorporating microparticles of blends of poly(DL-lactic-co-glycolic acid) (PLGA) and poly(ethylene glycol) (PEG) during a 26-week period in pH 7.4 phosphate buffered saline at 37°C. Using a fractional factorial design, four formulations of composite scaffolds were fabricated with varying PEG content of the microparticles, microparticle mass fraction of the composite material, and initial leachable porogen content of the scaffold formulations. PPF scaffolds without microparticles were fabricated with varying leachable porogen content for use as controls. The effects of including PLGA/PEG microparticles in PPF scaffolds and the influence of alterations in the composite formulation on scaffold mass, geometry, water absorption, mechanical properties and porosity were examined for cylindrical specimens with lengths of 13 mm and diameters of 6.5 mm. The composite scaffold composition affected the extent of loss of polymer mass, scaffold length, and diameter, with the greatest loss of polymer mass equal to 15 ± 5% over 26 weeks. No formulation, however, exhibited any variation in compressive modulus or peak compressive strength over time. Additionally, sample porosity, as determined by both mercury porosimetry and micro-computed tomography did not change during the period of this study. These results demonstrate that microparticle carriers can be incorporated into PPF scaffolds for

* This chapter has been submitted for publication as: E.L. Hedberg, C.K. Shih, J.J. Lemoine, M.D. Timmer, M.A.K. Liebschner, J.A. Jansen, A.G. Mikos, "In vitro degradation of porous poly(propylene fumarate)/poly(DL-lactic-co-glycolic acid) composite scaffolds," Biomaterials.
localized delivery of bioactive molecules without altering scaffold mechanical or structural properties up to 26 weeks *in vitro*.

1. Introduction

A wide variety of synthetic polymers are currently being explored for use as porous scaffolds for many tissue engineering strategies. For use in bone defect sites, polymer scaffolds must possess a number of specific properties in order to be successful. First, the material must be biocompatible to avoid an irresolvable inflammatory response that might lead to host rejection and/or the formation of an impenetrable fibrous capsule around the implant. The material must also support cellular migration and proliferation to allow for the infiltration of osteoblasts and the formation of bone. Structurally, the polymer scaffold must initially possess porosity and pore size distribution that is sufficient for cellular infiltration as well as the transport of nutrients into and cellular waste products out of the scaffold. In addition, the construct should possess mechanical properties similar to the surrounding host tissue in order to provide structural stability to a defect site without absorbing all of the mechanical stresses, which would result in stress shielding of the natural tissue and its eventual resorption. Furthermore, as the scaffold only serves as temporary support until bone is formed within the defect site, the ability to breakdown gradually into nontoxic degradation products either through hydrolysis or enzymatic degradation is also important. Ideally, the polymer scaffold would degrade at a rate equal to the rate of tissue ingrowth, allowing for maintenance of the scaffold structure and mechanical support during the early stages of tissue formation [1-4].

The aliphatic polyester poly(propylene fumarate) (PPF) is a synthetic material that is recently being explored for use as a degradable scaffold for bone tissue engineering. PPF consists of repeating units that contain one unsaturated double bound
that permits covalent crosslinking and two ester groups that allow for hydrolysis of the polymer into the nontoxic degradation products of fumaric acid and propylene glycol [5, 6]. An advantage of PPF over many other biodegradable, synthetic polymers is that it can be utilized as an injectable system, allowing for direct application into a defect site and cross-linking in situ. For use as an injectable scaffold, PPF can be crosslinked with the crosslinking agent poly(propylene fumarate)-diacrylate (PPF-DA) through free radical polymerization after addition of a thermal initiation system consisting of benzoyl peroxide and N,N-dimethyl-p-toluidine. A leachable porogen such as sodium chloride can be included for the creation of a highly porous network.

Recent studies with PPF have demonstrated that crosslinked PPF is able to support bone formation both in vivo and in vivo [7-9]. In vivo studies have also shown, however, that PPF does not induce bone formation in areas where the body would not normally form bone [10]. To create a potentially osteoinductive scaffold, osteogenic peptides or proteins can be incorporated into PPF scaffolds through the use of poly(DL-lactic-co-glycolic acid) (PLGA) microparticles. The use of the microparticle protects the bioactive factor during crosslinking of the PPF as well as offers more variables for the attenuation of the release kinetics of the factor from the scaffold. Previous studies have shown that the osteogenic peptide TP508 (Chrysalin®; MW = 22,311; AGYKDEGKRGDACEGDSGPFV) can be incorporated and released from these PPF/PLGA composite scaffolds and the release of TP508 can be controlled through alteration of the scaffold formulation [11].

This study examines the in vitro degradation of PPF/PLGA composite scaffolds. Scaffold mass, geometry, morphology, mechanical properties, and pore volume were examined over the course of 26 weeks to address the following questions: (1) How does the inclusion of PLGA microparticles into PPF scaffolds effect the scaffold material degradation? and (2) How do alterations in the PLGA microparticle formulation, the
PLGA to PPF ratio, and the initial leachable porogen content influence the composite material degradation?

2. Materials and Methods

2.1 Polymer Synthesis

2.1.1 PPF Synthesis

PPF was synthesized using a two-step process as previously described [11]. Briefly, diethyl fumarate (Acros Chemical, Pittsburgh, PA), propylene glycol (Acros), and zinc chloride (Fisher Chemicals, Pittsburgh, PA), were combined (molar ratio of 1:3:0.01, respectively) in a three-neck round bottom flask. The contents of the flask were mixed vigorously with an overhead mechanical stirrer and maintained under a nitrogen blanket at atmospheric pressure for approximately eight hours. The vapor temperature above the reaction was kept at approximately 10°C above the theoretical boiling point of ethanol (78°C) in order to remove this byproduct through distillation. The mixture was then trans-esterified through reducing the pressure (<1 mmHg) and maintaining the vapor temperature above the reaction at approximately 10°C above the theoretical boiling point of propylene glycol (48°C at 1 mmHg) in order to remove this byproduct through distillation. PPF was purified by dissolving the reaction mixture in methylene chloride and washing with aqueous hydrochloric acid (5%), water (x2), and brine (x2). After removing residual water with sodium sulfate (Fisher), the polymer was dried through rotary evaporation and vacuum drying. The final product was a clear, light yellow, viscous liquid with a number average molecular weight (Mn) 2,180 and a
polydispersity index (PI) 1.74, as determined by gel permeation chromatography compared to polystyrene standards. PPF was stored at -20 C until use.

2.1.2 PPF-DA Synthesis

PPF-DA was synthesized as previously described [11]. Briefly, fumaric acid, propylene glycol, and pyridine (Aldrich Chemical, Milwaukee, WI), were combined (molar ratio of 1:3:0.01, respectively) with 2-butanone (Aldrich) in a three-neck round bottom flask. The contents of the flask were mixed vigorously with an overhead mechanical stirrer and maintained under a nitrogen blanket at 85°C and atmospheric pressure. After approximately 18 hours, propylene glycol and 2-butanone were distilled off (80°C, atmospheric and reduced pressure, respectively). The reaction mixture was purified by washing with aqueous sodium hydroxide (5%) and brine. After removing residual water with sodium sulfate, the product, di-(2-hydroxypropyl) fumarate (diester), was dried through rotary evaporation and vacuum drying.

In the second reaction, acryloyl chloride (Aldrich) was slowly added drop-wise to a solution of diester and anhydrous triethylamine (Aldrich) in methylene chloride so the reaction temperature remained at approximately -5°C. The molar ratio of diester, acryloyl chloride, and triethylamine was 1:3:3, respectively. After the addition of the acryloyl chloride, the reaction was stirred overnight at room temperature. The triethylamine salt byproduct was filtered off and the methylene chloride solvent was rotary evaporated off. The product was extracted from the residue with excess ethyl ether. The solution was then washed with aqueous sodium hydroxide (5%), water, and brine. After removing residual water with sodium sulfate, the PPF-DA was dried through rotary evaporation and vacuum drying. Finally, the PPF-DA was suspended in a mixture of ethyl ether and petroleum ether (1:2 by volume) and further purified by elusion through a 7-inch silica gel column. Dried PPF-DA was stored at -20 C until use.
2.2 Microparticle Fabrication

Two formulations of microparticles using PLGA 50:50 (Medisorb®, Alkermes, Cincinnati, OH) were fabricated through a double-emulsion, solvent extraction technique (water-in-oil)-in water) as previously described [11]. Briefly, 125 μL ddH₂O were injected into a flint glass tube containing 237.5 g polymer (237.5 mg PLGA or 225.0 PLGA and 12.5 poly(ethylene glycol) (PEG, Mn=10,700, Aldrich) in 1 mL dichloromethane. The mixture was emulsified on a vortexer (Vortex Genie 2, Scientific Industries, Bohemia, NY) for 1 min prior to the addition of 1.5 mL of a 0.3% aqueous poly(vinyl alcohol) (PVA, 88 mol% hydrolyzed, Mn=13,000-23,000, Aldrich) solution and another minute of vortexing. This emulsion was then added to beakers containing 100 mL of 0.2% aqueous isopropanol and 98.5 mL of 0.3% aqueous poly(vinyl alcohol) and stirred rapidly for 1 h. Microparticles were collected through centrifugation at 180 × g for 1 min, lyophilized to dryness, and stored at -40°C until use. No bioactive molecules were loaded into the microparticles.

2.3 Experimental Design

To evaluate the properties of degrading PPF/PLGA composite scaffolds, six sample formulations were employed in this study. Four formulations composed of PPF, PPF-DA (crosslinking molecule), PLGA/PEG microparticles, and NaCl (leachable porogen, sieved to 200 to 500 μm) were fabricated according to a Resolution III two-level fractional factorial design [12, 13]. In this study, three variables were considered: 1) PEG content within the PLGA microparticles, 2) the microparticle content in the composites, and 3) the NaCl initial mass percent of the composites. High and low values were chosen for each parameter, and these levels were combined according to the fractional factorial design to create four composite formulations. The values for all
parameters and combinations for all formulations are presented in Table 4-1(a) and Table 4-1(b), respectively. The remaining two formulations were non-microparticle loaded PPF scaffold controls at the high and low NaCl levels (75 and 80 wt%).

2.4 Sample Preparation

In a typical procedure for the preparation of composites using PPF-DA as a crosslinker, 1.0 g PPF was mixed with 2.05 g PPF-DA (the double bond ratio of PPF/PPF-DA was 0.5) in the presence of CH₂Cl₂ and stirred overnight at room temperature. After removal of the solvent, 0.14 mL of initiator solution in diethyl fumarate (0.1 g benzoyl peroxide (Aldrich Chemical) per 1 mL diethyl fumarate) was added to the PPF/PPF-DA mixture and agitated thoroughly. Proper amounts of microparticles and NaCl were each added to the mixture and agitated until they appeared to be homogeneously distributed. Finally, 5 μL of N,N-dimethyl-p-toluidine (Aldrich Chemical) was injected into the mixture with vigorous mixing. The material was inserted into glass cylindrical vials (diameter = 6.5 mm) and placed in a 37°C oven for 20 min. The crosslinked samples were removed from the molds and cut to a length of 13 mm.

2.5 In Vitro Degradation

Each specimen was placed in an individual glass vial and immersed in approximately 20 ml phosphate buffered saline (PBS). The vials were stored in a 37°C environmental chamber on a shaker table (70 rpm). The buffer was exchanged every 8 h for the first 48 h, daily for the next 5 days, and weekly for the remaining 25 weeks. The solution pH for each sample group was recorded in triplicates (n = 3) prior to every buffer exchange. Measurements were also taken for buffer solutions that did not have
submerged specimens \((n = 18)\) in order to evaluate the change in medium pH due to polymer degradation. At 4 days, 2, 8, 12, 18, and 26 weeks specimens were removed to undergo gravimetric and dimensional analysis, mechanical testing, micro-computed tomography, mercury porosimetry, and imaging.

2.6 Gravimetric and Dimensional Analysis

Prior to emersion in PBS, the initial scaffold mass \((m_{b,i})\), length \((l_i)\), and diameter \((d_i)\) of all samples was measured. At specified timepoints, three specimens were removed from the buffer solutions and the external surface was blotted dry with a piece of wax paper. The wet samples were weighed \((m_w)\) and their length \((l_w)\) and diameter \((d_w)\) was measured. Subsequently, samples were vacuum dried for 48 h. The dry mass \((m_d)\) length \((l_d)\), and diameter \((d_d)\) of all samples was measured. The initial polymer mass \((m_{p,i})\) was determined by multiplying the initial samples mass \((m_{b,i})\) by the initial polymer mass fraction where the initial polymer fraction is 1 minus the initial NaCl mass fraction. The fractional polymer mass remaining \((m_d/m_{p,i})\), fractional length remaining \((l_d/l_i)\), fractional diameter remaining \((d_d/d_i)\), and water absorption \(((m_w-m_d)/ m_d)\) were calculated for each sample at each timepoint \((n=3)\). Dry samples were then hemisectioned along the cross-section to create two cylindrical specimens of approximately 6.5 mm in height and 6.5 mm in diameter. One half of each sample was used for porosity measurements and the other for imaging via scanning electron microscopy.

2.7 Mechanical Testing

Compressive mechanical testing was conducted in accordance to ASTM D695-96 with an 858 Material Testing System mechanical testing machine (MTS System Corporation, Eden Prairie, MN) on wet samples after removal from PBS \((n = 5)\). The cylinders were compressed between two plates moving at a crosshead speed of 1
mm/min until failure, while load and displacement was recorded throughout. Based on the sample geometry, the stress versus strain behavior was obtained and plotted. The peak compressive strength and the compressive modulus were determined form the stress-strain curve using Testworks 4 Analysis Software (MTS Systems Corporation).

2.8 Micro-Computed Tomography

Before analysis of the samples began, a thresholding analysis was conducted using porous PPF/PLGA scaffolds to optimize the equipment test conditions for quantification of PPF/PLGA composite porosity [14]. Briefly, the threshold value was varied between 15 and 55 and the change in porosity to sample volume ratio was measured. A minimum change in porosity with varying threshold level suggested the optimal threshold value of 35 for this study. For one run of the μCT (Scanco Medical μCT 80, Scanco Medical, Bassersdorf, Switzerland), five vacuum dried specimens were fixed in place vertically within the sample holder and then placed in the sample chamber. Before the complete scan, single 2-D x-ray scan was performed in order to determine the position of the samples within the chamber. Complete scans (energy = 50 kV; intensity = 80 μA; isotropic resolution 10 μm) were conducted over a length of 2.5 mm beginning 3.0 mm from the uncut end of the sample in order to eliminate end effects from this analysis. To create reconstructions, a long integration time (300 ms) was used because of the low x-ray attenuation coefficient of polymeric materials. The Scanco μCT Evaluation Program was used to calculate individual sample total porosity and surface area density using a reconstruction radius 2 mm inside the cylindrical samples (radius = 3.25 mm) in order to eliminate potential edge effects.
2.9 Mercury Intrusion Porosimetry

After analysis by μCT, the scaffold void volume (porosity) attributed to an interconnected pore network was determined using mercury porosimetry (Autoscan-500, Quantachrome, Boyton Beach, FL) to determine. Each specimen was loaded into the porosimeter intrusion chamber and the chamber was evacuated to 0.2 psi. Mercury was loaded into the chamber to 0.5 psi and intruded into the sample by increasing the pressure from 0.5 to 500 psi. The intruded volume and pressure were monitored during each run. The porosimeter measurement yielded the intruded volume of mercury per gram of sample, which was assumed to be equal to the pore volume ($V_{por}$) per gram sample. Sample porosity ($\varepsilon$) was determined by the equation:

$$\varepsilon = \frac{V_{por}}{V_{por} + (1/\rho)}$$

where $\rho$ is the sample density. In calculating $\rho$, the initial mass loss is assumed to be due solely to the effects of salt leaching, so the mass loss exceeding a sample’s theoretical salt content is attributed only to polymer degradation. Accordingly, NaCl content ($C_s = g$ NaCl/total g), NaCl density ($\rho_s = 2.17$ g/ml), polymer content ($C_p = g$ polymer/total g), and polymer density ($\rho_p = 1.28$ g/ml) [15]were used to calculate the sample density ($\rho$) from the equation

$$\rho = (C_s + C_p)/(C_s/\rho_s + (C_p/\rho_p))$$

2.10 Scanning Electron Microscopy

At day 4 and week 26, pore morphology and composite composition of the composite scaffolds were imaged by scanning electron microscopy. Specimens were placed on sample holders with the cut, scaffold interior surface facing up. Samples were then coated with gold for 1 min at 99 mA using a CrC-100 Sputter System (Torr
International, New Windsor, NY). The top surfaces of the samples were viewed under a JSM-5300 SEM (Jeol, Boston, MA) operated at 15-20 kV.

**Statistical Analysis**

Results are reported as means ± standard deviation. Data analysis was performed using GraphPad InStat version 3.05 statistical software (San Diego, California, USA). To determine differences between time-points for each formulation as well as between formulations at each timepoint, a non-parametric analysis of variance (Kruskal-Wallis Test) was first performed to determine statistical significance (p < 0.05) within each data set. When the analysis of variance detected significance, a Dunn's Multiple Comparison Test was run with a confidence level of 95%.

4. Results

3.1 pH

To evaluate the degradation of the scaffolds, the medium pH for the specimens was compared to buffer that was held under the same conditions but did not contain any samples (blanks). A storage buffer with a lower pH that the blank buffer would indicate the release of acidic products from the scaffolds. Figure 4-1 shows the difference in pH between the storage and blank buffer solutions for each scaffold formulation. At 8h, an initial decrease in storage pH was observed. By 16 h the difference between the storage and the buffer pH was negligible and remained so through week 2. At week 2, the storage pH solution for composite formulations with high microparticle contents (C3 and C4) began to decrease. This decrease in pH persisted through week 8, with a maximum deviation from storage pH of −0.47 ± 0.6 at week 3 for C3 and −0.37 ± 0.12 at week 5 for C4. A smaller maximum decrease in pH was observed for composite formulations with
low microparticle contents (-0.17 and -0.13 for C1 and C2, respectively), while no
decrease in pH was observed for blank scaffold formulations (B1 and B2) during the
same time period. The pH did not decrease again for any formulation over the
remaining 18 weeks of the study.

3.2 Gravimetric and Dimensional Analysis

Fractional change in polymer mass, change in length, and change in diameter
were calculated to assess the amount of material lost from the scaffold formulations over
the course of 26 weeks in vitro. Fractional change in polymer mass results are
presented in Figure 4-2. These data show that formulation C4 was the only formulation
to decrease in polymer mass over the course of the study. C4 scaffolds exhibited a
gradual loss of polymer mass, with a statistically significant difference (p < 0.05)
between day 4 (1.03 ± 0.03) and week 26 (0.85 ± 0.05).

Results from fractional change in length are shown in Figure 4-3. For this
parameter, formulations C3 and C4 displayed a statistical decrease (p < 0.05) between
day 4 and week 26. The fractional change in diameter results (Figure 4-4) show a slight
decrease in the sample diameters for both formulations C3 and C4 by week 26 with the
difference between day 4 and day 26 significant (p < 0.05) for formulation C3 only.
Although statistical significance was detected for these parameters, the changes were
minimal (less than 6%) and can be attributed to experimental error.

To evaluate the water content of the scaffold material, water absorption was
determined (Figure 4-5). Day 4 water absorption results varied from 1.06 to 1.55 for the
composite formulations and from 1.01 to 1.58 for blank formulations. Leaching of the
porogen was expected to be complete by day 4, while polymer degradation was
expected to be minimal. Formulations C1, C3, C4, and B1 all exhibited statistical (p <
0.05) increase in water absorption over time.
3.3 Mechanical Testing

Compressive properties of wet scaffolds were determined following removal from buffer solutions (Figure 4-6). Initial values for compressive modulus and peak stress follow similar trends, with low initial porogen content formulations (C2, C3, and B1) generally exhibiting higher values than high initial porogen content formulations (C1, C4, and B2). Over time, no variation in either property was observed.

3.4 Micro-Computed Tomography

μCT conducted at a resolution of 10 μm was employed to determine the total pore volume fraction (porosity) and surface area density of scaffold formulations as well as create three-dimensional reconstructions of the scaffolds’ internal architectures. Porosity results are presented in Figure 4-7. No significant differences were detected either between formulations for any time point or between time-points for any formulation. Similar results were obtained for the surface area density calculations (data not shown). Three-dimensional images of representative composite scaffolds at day 4 and week 26 are presented in Figure 4-8.

3.5 Mercury Porosimetry

Another method for determining the scaffold porosity is through the use of mercury intrusion porosimetry. Where μCT determines the total pore volume fraction, mercury intrusion porosimetry measures only the pore volume associated with an interconnected pore network. Results for mercury intrusion porosimetry can be found in Figure 4-9. As with the μCT results, no significant differences were detected either between formulations for any time-point or between time-points for any formulation.
3.6 SEM Imaging

Scanning electron microscopy was used to obtain images of the internal architecture of the scaffold formulations. Representative images displaying the overall scaffold morphology of the composite scaffolds can be found in Figure 4-10. Images indicate retention of pore shape over the course of 26 weeks. At greater magnification, PLGA microparticles embedded in the PPF were evident at both day 4 and week 26 (Figure 4-11). Comparison of fractured microparticles shows that although the microparticles are still physically intact at week 26, the polymer of week 26 microparticles is more disrupted than the polymer of day 4 microparticles.

4. Discussion

Recent studies in our laboratory have examined the degradation of solid PPF networks fabricated through the crosslinking of PPF with the crosslinking agent PPF-DA through thermal initiation. Results from these experiments demonstrated that this material exhibits minimal degradation after 26 weeks in vitro as determined by gravimetric and dimensional analysis and mechanical testing [16]. The study presented here explores the effect of the addition of PLGA microparticles to the polymer phase of porous PPF scaffolds as well as the effects of altering scaffold formulation parameters such as the PLGA microparticle formulation, the PLGA to PPF ratio, and the initial leachable porogen content on the PPF/PLGA composite scaffold degradation over the course of 26 weeks in vitro.

PPF and PLGA are both synthetic polyesters that degrade through hydrolysis of their ester linkages. Their initial rate of degradation is primarily dependent on two major processes, the diffusion of water into the polymer bulk and the random chain scission of the polymer backbones [17, 18]. The rate of these two processes can vary depending
on a number of polymer and device parameters. For instance, decreasing the
crosslinking density of PPF networks has been shown to increase the \textit{in vitro} material
degradation [16, 19], and decreasing the molecular weight of PLGA decreases its rate of
degradation [20]. Device dimensions such as size, shape and surface area to volume
also play a role in regulating the rate of water ingress into the polymer network as well
as removal of autocatalytic degradation products out of the polymer network, thereby
influencing many stages of polymer degradation [20].

Previous studies have demonstrated that PLGA degrade much faster than PPF.
PLGA/PEG microparticles fabricated using PLGA with a lactic to glycolic acid ratio of 1:1
and a PEG content of 5\% were found to have an average half-life of 14.8 days when
stored in buffer at pH 7.4 [21]. Additionally, PLGA/PEG microparticles were found to
degradate faster (half-life of 5.5 days) at pH 3.0. In PPF/PLGA composites the PLGA
microparticles were expected to degrade faster than the surrounding PPF leading to an
increase in the porosity of the composite scaffolds. Calculating the theoretical volume
fraction of the microparticles using the measured microparticle density of 0.5 g/mL
yielded volumes of approximately 5 mL microparticle/mL sample for the low microparticle
loaded formulations (0.03 g microparticles/g polymer) and 25 mL microparticle/mL
sample for the high microparticle loaded formulations (0.09 g microparticles/g polymer).
While the theoretical increase in porosity due to complete degradation of the PLGA
microparticles was too small to detect due to experimental variability, the increase for the
high microparticle loaded formulations was large enough to be detectable with both
techniques employed here.

Two different methods were utilized to measure the porosity of the samples,
mercury porosimetry and \textit{\mu}CT. Mercury porosimetry is a well-documented technique
that uses a steady increase in pressure in a closed chamber containing the sample to
push mercury slowly into the sample porosity. The volume of mercury that enters the
sample is measured and is considered to be equal to the sample void volume. This technique allows for determination of the void volume only of an interconnected pore structure, as the mercury cannot access isolated pores. For PPF/PLGA composites, pores formed through the degradation of microparticles completely embedded in the PPF can not be detected with this method, leading to an underestimation of the void volume of the sample. Additionally, the presence of large pores at the surface of a sample can lead to further underestimation of the porosity as these pores may fill with mercury during chamber conditioning prior to void volume determination.

µCT is a relatively new technique that is beginning to find application in polymer scaffold characterization [14, 22-24]. µCT is a fast, non-destructive method that utilizes X-rays to create multiple 2-dimentional images of a sample. With these images, analytical software is used to reconstruct the complete three-dimensional structure of a sample as well as calculate sample void volume, surface area density, pore wall thickness, and pore edge length [14]. Although the resolution of our reconstructions was smaller than the average microparticle size, PLGA microparticles could not be imaged independently of the PPF due to their similar x-ray attenuation coefficients. Because of this, the results represent the polymer of the composite formulations as a single continuous material. Unlike with mercury porosimetry, the results represent the total void volume, and not just the volume of the interconnected pores. This technique, therefore, should detect the change in porosity attributed to the degradation of PLGA microparticles embedded in PPF. Again, however, no increase in porosity was detected over time. The lack of an increase in the measured porosity may be attributed to incomplete degradation of the microparticles. SEM images of week 26 samples evidence of intact PLGA microparticles at week 26.
The degradation products of both PPF and PLGA are acidic [5, 20]. The deviation of buffer pH results show a decrease in the pH in the sample vials from week 2 to week 8, indicating the release of acidic products from the samples during this period. Composite scaffolds loaded with the highest concentration of microparticles (C3 and C4, 0.1 g MP/g polymer) exhibited the greatest drop in pH during this time period, with C1 and C2 (0.03 g MP/g polymer) exhibiting a smaller change in pH, and the unloaded scaffolds (B1 and B2) exhibiting minimal change. The timing of the drop in pH along with its correlation with microparticle loading suggest that degradation of the PLGA microparticles did occur. Additionally, a statistically significant change in polymer mass was suggest that the change in pH is attributed to the loss of PLGA microparticles and not PPF.

It was hypothesized that the degradation of the PLGA microparticles would influence the degradation of the surrounding PPF. The presence of lactic and glycolic acid within the scaffold would lead to a decrease in local pH, accelerating the degradation of the PPF, which has been shown to degrade faster in acidic environments[16]. PPF undergoes bulk degradation and an early indication of degradation of bulk degrading materials is a decrease in crosslinking density [16]. As the mechanical properties of PPF materials have been shown to be dependent on the crosslinking density of the network [16], scaffold compressive modulus and peak strength were measured. No statistical decrease in these parameters was detected for any formulation, indicating that the PPF crosslinked network is still intact regardless of composite formulation.

Gravimetric and dimensional analysis results support the mechanical testing data. Minimal differences were observed between day 4 and day 26 data for fractional change in polymer mass, length and diameter for most scaffold formulations. Significant differences in these parameters were detected for one formulation, C4. These changes,
however, were most likely due to mechanically induced fragmentation of the weakest scaffold formulation (see 4-6) during the course of the experiment and not polymer degradation.

Water absorption was measured in order to assist in assessment of the breakdown of the PPF scaffold. Previously studies conducted in our laboratory have demonstrated a marked increase in water absorption coincident with a decrease in sample mass and an increase in sample dimensions [25]. In the study presented here, neither swelling of the scaffolds nor a dramatic decrease in mass was observed for any formulations. Increases in water absorption, however, did occur. These increased can be attributed to the mechanism of polymer absorption into the scaffolds. PPF is a highly hydrophobic material and the water absorption both into the pores of the scaffold and into the polymer network is slow. As the porogen leached out of the scaffolds during the first few days in PBS, the pores of the scaffolds filled with air bubbles as well as water. The presence of these air bubbles persist within the inner pores of the scaffold, blocking the ingress of water. As equilibrium water content is not reached, water content varies over time. Therefore, variation in the water absorption without the corresponding changes in mass, dimensions and/or mechanical properties does not suggest degradation of the polymer scaffolds.

5. Conclusions

Although degradation of the PLGA microparticles was observed, the PLGA microparticle loaded PPF scaffolds showed no difference in the extent of degradation compared to unloaded controls. Additionally, alteration in composite parameters such as the PLGA microparticle formulation, the PLGA to PPF ratio, and the initial leachable porogen content were not observed to influence the degradation through 26 weeks in vitro. These results demonstrate that microparticle carriers can be incorporated into
PPF scaffolds for localized delivery of tissue morphogens without altering the expected rate of degradation compared to blank, unloaded PPF scaffolds.
### Table 4-I: PPF/PLGA Degradation Experimental Design

(a) High and Low Levels for Parameters Tested in the Resolution III, Two-Level Fractional Factorial Design

<table>
<thead>
<tr>
<th></th>
<th>g PEG/ g Microparticle(^a)</th>
<th>g Microparticle/ g Composite Polymer(^b)</th>
<th>g NaCl / g Composite(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Level (+)</td>
<td>0.05</td>
<td>0.09</td>
<td>0.85</td>
</tr>
<tr>
<td>Low Level (-)</td>
<td>0.00</td>
<td>0.03</td>
<td>0.70</td>
</tr>
</tbody>
</table>

(b) Combinations of the Experimental Values in the \(2^{3-1}\) Design (C1-C4) and Unloaded Controls (B1 and B2)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>g PEG/ g Microparticle(^a)</th>
<th>g Microparticle/ g Composite Polymer(^b)</th>
<th>g NaCl / g Composite(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C2</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C3</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B1</td>
<td>N/A</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>B2</td>
<td>N/A</td>
<td>N/A</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\) Microparticle comprised of PLGA and PEG  
\(^b\) Composite polymer comprised of PPF, PPF-DA, and microparticles  
\(^c\) Composite comprised of PPF, PPF-DA, microparticles, and NaCl
Figure 4-1: Deviation of solution pH from buffer pH blanks as a function of degradation time for PPF/PLGA composite scaffolds (C1, C2, C3, C4) and unloaded PPF scaffolds (B1, B2). Results are presented as means ± standard deviation for n = 3.
Figure 4-2: Fractional change in polymer mass during in vivo degradation for PPF/PLGA composite scaffolds (C1, C2, C3, C4) and unloaded PPF scaffolds (B1, B2). Results are presented as means ± standard deviation for n = 4.
Figure 4-3: Fractional change in length during *in vivo* degradation for PPF/PLGA composite scaffolds (C1, C2, C3, C4) and unloaded PPF scaffolds (B1, B2). Results are presented as means ± standard deviation for n = 4.
Figure 4-4: Fractional change in diameter during *in vivo* degradation for PPF/PLGA composite scaffolds (C1, C2, C3, C4) and unloaded PPF scaffolds (B1, B2). Results are presented as means ± standard deviation for *n* = 4.
Figure 4-5: Sample water absorption during *in vivo* degradation for PPF/PLGA composite scaffolds (C1, C2, C3, C4) and unloaded PPF scaffolds (B1, B2). Results are presented as means ± standard deviation for n = 4.
Figure 4-6: Compressive modulus (a) and peak stress (b) during in vivo degradation for PPF/PLGA composite scaffolds (C1, C2, C3, C4) and unloaded PPF scaffolds (B1, B2). Results are presented as means ± standard deviation for n = 5.
Figure 4-7: Porosity during *in vivo* degradation for PPF/PLGA composite scaffolds (C1, C2, C3, C4) and unloaded PPF scaffolds (B1, B2) as determined by micro-computed tomography. Results are presented as means ± standard deviation for \( n = 3 \).
Figure 4-8: Micro-computed tomography created images of PPF/PLGA scaffolds at day 4 and week 26. (a.) C1, day 4; (b.) C2, day 4; (c.) C3, day 4; (d.) C1, week 26; (e.) C2, day 26; (f.) C3, week 26. Sampled area was 4 mm in diameter and 2.5 mm in thickness with a special resolution of 10 μm. Bars represent 1 mm.
Figure 4-9: Porosity during *in vivo* degradation for PPF/PLGA composite scaffolds (C1, C2, C3, C4) and unloaded PPF scaffolds (B1, B2) as determined by mercury intrusion porosimetry. Results are presented as means ± standard deviation for n = 3.
Figure 4-10: Scanning electron micrographs of porous PPF/PLGA composite scaffolds. C1, day 4 (a) and week 26 (b); C2, day 4 (c) and week 26 (d); C3, day 4 (e) and week 26 (f); C4, day 4 (g) and week 26 (h).
**Figure 4-11:** Scanning electron micrographs of PLGA microparticles embedded in PPF (formulation C4) at day 4 (a) and week 26 (b).
CHAPTER V

Effect of Varied Release Kinetics of the Osteogenic Thrombin Peptide TP508 from Biodegradable, Polymeric Scaffolds on Bone Formation In Vivo

Abstract

This study was designed to assess the influence of varied release kinetics of the osteogenic thrombin peptide TP508 from osteoconductive poly(propylene fumarate)-based (PPF) composite scaffolds on bone formation in vivo. Four classes of scaffolds were constructed with different dosages (200, 100, or 0 μg) and release kinetics (large burst release, minimal burst release, or no release) and implanted in 15.0 mm segmental defects in rabbit radii. The animals were euthanized at 12 weeks and the implants were analyzed by light microscopy, histological scoring analysis, and histomorphometric analysis. Samples from all classes displayed bone growth within the pores of the scaffold near the edges of the defect. In areas where bone was not observed, the pores were filled with mostly fibrous tissue and exhibited minimal inflammatory response for all classes. In contrast to other scaffold classes, scaffolds containing a total dose of 200 μg TP508 and exhibiting a large burst release profile showed a statistically more bone formation guided along the surface of the scaffold, with these scaffolds averaging 80% of the defect length bridged with bone compared to 10% or less bridged for the other scaffold classes. These results demonstrate that the extent of in vivo bone formation in response to controlled release from PPF-composite scaffolds is determined by the release kinetics of the incorporated osteogenic peptide.

1. Introduction

The current gold standard for the repair of non-union bone defects includes the use of autologous and/or allografts to replace damaged tissue. Limitations in supply, donor site morbidity, and pathogen transfer are difficulties that limit the success of these strategies [1]. Recently, researchers from medicine, biology, and engineering have come together in the field of tissue engineering in an attempt to overcome some of the shortcomings associated with these current methods of bone repair.

One area of focus in tissue engineering is the delivery of osteogenic factors in an attempt to modulate the formation of bone. The most widely explored osteogenic factors are the members of the transforming growth factor-β (TGF-β) super-family that include transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein-2 (BMP-2). These proteins have all been shown to augment the bone forming capacity of osteoblastic cell populations when delivered at the appropriate times in the wound healing cascade [2-4]. Unfortunately, these factors have been shown to be a challenge to formulate and deliver due to their complex tertiary structures, short biological half-lives, and possible systemic toxicity [5].

Osteogenic peptides offer an advantage over the proteins discussed above as they are often less costly to produce and less likely to lose bioactivity during storage and delivery due to their short, linear structure. An example of an osteogenic peptide is TP508 (Chrysalin®, MW = 2,311; AGYPDEGKRGDACEGDSGPFV). TP508 is a 23 amino acid peptide representing the non-proteolytic receptor-binding domain of human prothrombin. Thrombin is known to activate blood platelets, to promote angiogenesis, to support inflammatory cell adhesion and to increase vascular permeability, which are all very relevant for the final wound healing process. Research on TP508 began with the exploration of its ability to support wound healing in dermal tissue through promotion of
revascularization of wound sites [6-9]. More recently, TP508 has also been examined as a potential candidate to support bone healing in fresh fractures and segmental defects [10, 11]. These studies suggest that TP508 accelerates wound healing by enhancing the recruitment of inflammatory cells.

In order to efficiently deliver the osteogenic molecules \textit{in vivo}, these factors can be incorporated into a carrier that can be implanted directly into a defect site. This method results in localized drug delivery and reduces possible toxic systemic effects. In addition, through proper selection of the carrier material, the rate of release of the entrapped factors can be controlled to meet the temporal requirements of the wound healing response. Synthetic polymers are attractive for this application as they can be fabricated to exact specification allowing for the fine-tuning of the physical properties that influence drug release such as the mesh size of their crosslinked networks as well as their rate of degradation. The poly(\(\alpha\)-hydroxy acid) family of polymers including poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymers poly(D,L-lactic-co-glycolic acid) (PLGA), have been extensively explored for bone tissue engineering applications as they are biocompatible, undergo controllable hydrolytic degradation into natural metabolites, and can be processed into many forms [12-14]. Microparticles of PLA and PLGA have been used to deliver many factors including TGF-\(\beta\)1 and BMP-2 into osseous defects [13]. In order to provide a support for cellular migration, PLGA scaffolds have also been developed. Some of this work focused on the adsorption of therapeutic agents onto prefabricated scaffolds, but control of the factor’s release kinetics was found to be limited with this technique. For controlled release, osteogenic factors can be incorporated directly into the polymer component of poly(\(\alpha\)-hydroxy acid) matrices through a number of techniques [15-18] and their final release can be modulated by parameters such as pore size and protein loading of the matrix [17].
Another synthetic polymer that is currently under investigation for bone tissue engineering is the linear polyester poly(propylene fumarate) (PPF). PPF contains many unsaturated double bonds for crosslinking via free radical propagation using either photo or thermally induced polymerization. In its uncrosslinked form, PPF is a viscous liquid that can be mixed with an initiator solution and injected into a mold to form a prefabricated scaffold or directly into a defect site and allowed to cure in situ. As an injectable, PPF reduces the surgical manipulation required for implantation by filling irregularly shaped defect. As the crosslinking of PPF occurs with minimal heat release, minimal damage to the surrounding tissue is expected [19]. Additionally, crosslinking of PPF can be tailored to occur anywhere from 2 to 20 minutes after addition of the initiator solution depending on the needs of the surgeon [19]. A leachable porogen can be included into the injected polymer mixture, resulting in a highly porous polymer scaffold upon dissolution of the porogen. In vitro studies have demonstrated that PPF is able to support pre-osteoblast-like cell migration, differentiation, proliferation, and extracellular matrix production [4, 20]. PPF implanted in cranial defects in the rabbit have shown that PPF supports bone growth in vivo, but does not induce its formation [21]. Furthermore, PPF contains ester linkages that render the material degradable through hydrolytic cleavage of these bonds. The rate of degradation of PPF-based scaffolds has been shown to be dependent on parameters such as the PPF crosslinking density and the initial scaffold porosity [22, 23].

However, several challenging issues must be overcome before PPF can be applied as an injectable bone material for bone tissue engineering. For example, to incorporate biologically active molecules into PFF scaffolds, PLGA microparticles loaded with a specific bioactive factor can be embedded in the polymer phase of the PPF scaffold. The use of a microparticle carrier protects the bioactive factor from potentially adverse conditions during the PPF crosslinking and offers additional fabrication
parameters for the attenuation of the bioactive factor's release kinetics. Previous research has demonstrated that varying the formulation parameters of these PPF/PLGA composite scaffolds such as the drug loading of the PLGA microparticles, microparticle loading of the polymer scaffold, and porogen content of the composite material results in alterations of release kinetics of an entrapped molecule from these PPF/PLGA composite scaffolds [24, 25].

The study presented here sought to investigate the effects of varied release kinetics of the osteogenic peptide TP508 from a PPF/PLGA composite scaffold on bone formation. TP508 accelerates the recruitment of inflammatory cells to the wound site. Therefore, we hypothesized that the release kinetics of TP508 would affect the bone healing repair in large sized segmental defects. Specifically, we hypothesized that: (1) a PPF/PLGA composite scaffold that released 200 μg with a minimal initial burst release of TP508 would result in a greater amount of bone formation than the same dose delivered with a large initial burst release; and (2) the elimination of an initial burst release would necessitate a lower dose to induce bone formation. To address these hypotheses we investigated the bone forming capacity of four PPF/PLGA scaffold classes in a 15 mm segmental defect in the rabbit radius. The classes employed in this study include PPF/PLGA scaffolds that exhibited: (a) a large burst release of 200 μg TP508 (LB200); (b) a minimal burst release of 200 μg TP508 (MB200); (c) a minimal burst of 100 μg TP508 (MB100); and, (d) no release of TP508 (NR). Samples were retrieved 12 weeks post-implantation and assessed using histological and histomorphometric analyses.
2. Materials and Methods

2.1 PPF Synthesis

PPF was synthesized following a two-step procedure [25]. Briefly, 1 mole diethyl fumarate (Acros Chemical, Pittsburgh, PA) and 3 moles 1,2 propanediol (Acros Chemical) were reacted using ZnCl₂ (Fisher Chemical, Pittsburgh, PA) as a catalyst to form the intermediate bis(hydroxypropyl) fumarate. The reaction was held under a nitrogen blanket at atmospheric pressure. As the reaction by-product was ethanol, the vapor temperature above the reaction was maintained at approximately 10°C above the theoretical boiling point of ethanol (78°C) in order to remove the by-product through distillation. Bis(hydroxypropyl) fumarate was then transesterified at low pressure (<1 mmHg) producing PPF and 1,2 propanediol as a byproduct. The vapor temperature above the reaction was maintained at approximately 10°C above the theoretical boiling point of 1,2 propanediol (48°C at 1 mmHg) in order to remove the byproduct through distillation. The reaction continued until the product had the desired molecular weight as determined by gel permeation chromatography. To purify the PPF, the product was dissolved in methylene chloride (Fisher Chemical). The organic mixture was first washed with a 5% HCl solution to remove the catalyst and then twice with water and twice with brine. The organic phase was dried with sodium sulfate and the drying agent removed through vacuum filtration. Methylene chloride was removed from the PPF by rotary evaporation followed by vacuum drying. The final product was a clear, light yellow, viscous liquid with a number average molecular weight (Mₙ) 2,180 and a polydispersity index (PI) 1.74, as determined by gel permeation chromatography compared to polystyrene standards. PPF was stored at -20°C until use.
2.2 PPF-DA Synthesis

PPF-DA was synthesized in two-step procedure [25]. One mole fumaric acid (Aldrich Chemical, Milwaukee, WI) was reacted with 3 moles 1,2 propanediol (Acros Chemical) in the presence of 2-butanone (Aldrich Chemical) using pyridine (Aldrich Chemical) as a catalyst. The reaction was maintained at 85°C and atmospheric pressure for 14 to 18 hours. Fumaric acid is insoluble in 2-butanone so the reaction was considered complete upon dissolution of the white fumaric acid precipitate. The completed reaction mixture was heated to 80°C to distill off the excess 1,2 propanediol. When the 1,2 propanediol removal was complete, the pressure was decreased with aspiration to remove the 2-butanone. The remaining mixture was then washed once with a 5% NaOH solution and once with brine. The product, bis(hydroxypropyl) fumarate was dried with magnesium sulfate and vacuum dried. In the next step, 3 moles acryloyl chloride (Aldrich Chemical) were added drop-wise to 1 mole bis(hydroxypropyl) fumarate in the presence of 3 moles anhydrous triethyleamine (Aldrich Chemical) for 12 hours at -5°C and atmospheric pressure. The reaction mixture was dried under vacuum and PPF-DA was extracted through serial washings with ethyl ether (Fisher Chemical). For further purification, the PPF-DA was dissolved in methylene chloride, washed with base and brine, dried over magnesium sulfate, and filtered. Methylene chloride was removed through rotary evaporation. Finally, the PPF-DA was resuspended in a mixture of ethyl ether and petroleum ether (Fisher Chemical) (1:2 by volume) and further purified by passage through a 7-inch silica gel column. Dried PPF-DA was stored at -20°C until use.
2.3 Microparticle Fabrication

Microparticles of 50:50 PLGA (Medisorb®, Alkermes, Cincinnati, OH) loaded with TP508 (Chrysalis Biotechnology, Inc., Galveston, TX) were prepared by a double-emulsion, solvent extraction technique ((water-in-oil)-in water) as previously described [25]. Briefly, 125 μl of aqueous solution (15.0 mg TP508 in ddH2O) was injected into a flint glass tube containing the 235.0 mg PLGA in 1 mL dichloromethane. This mixture was emulsified on a vortexer (Vortex Genie 2, Scientific Industries, Bohemia, NY). The first emulsion was then combined with 1.5 mL 0.3% aqueous PVA solution (Aldrich Chemical) with vortexing, producing a second emulsion. This second emulsion was added to 100 mL of 0.2% aqueous isopropanol (Fisher Chemical) and 98.5 mL of 0.3% aqueous PVA with rapid stirring for 1 h. Microparticles were collected through centrifugation at 180 x g for 1 min, lyophilized to dryness, and stored at -40°C until use. The final product was spherical microparticles with a mass loading of 0.066 g TP508/g microparticle as determined by an established solvent extraction technique[26]. Blank microparticles were fabricated as outlined above with omission of the TP508 from the initial aqueous solution.

To achieve the proper dosage in each scaffold class without altering the proportion of microparticles to polymer in the composite scaffolds, TP508 loaded microparticles were combined with blank, unloaded microparticles. The mixtures prepared contained 44% and 22% loaded microparticles per mass for scaffolds containing 200 μg and 100 μg TP508, respectively.

2.4 PPF Composite Scaffold Fabrication

Four classes of PPF composite scaffolds were fabricated for this study (Table 5-1; Figure 5-1) [25]. For all classes, scaffolds were thermally crosslinked using the free
radical initiator benzoyl peroxide (BP; Aldrich Chemical). An initiator solution was prepared by dissolving 1 g BP in 10 mL diethyl fumarate. PPF was mixed with PPF-DA (the double bond ratio of PPF:PPF-DA was 1:2) in the presence of CH₂Cl₂ and stirred overnight at room temperature to ensure proper mixing of the two viscous solutions. After removal of the solvent from the polymer, the initiator solution (0.05 mL/g polymer) was added to the mixture with rapid stirring. For formulations in which the PLGA microparticles were located within the polymeric component of the scaffolds (MB200, MB100, and NR), microparticle mixtures (0.09 g microparticles/g polymer) were mixed in until homogeneously dispersed. All formulations then received 4 g of the water soluble porogen NaCl per gram of the polymer-microparticle mixture. Finally, the resulting paste was placed on a Teflon plate and 1.6 µl N,N-dimethyl-p-toluidine (DMT) per gram of polymer was kneaded into the mixture. The material was inserted into Teflon molds (diameter = 4.3 mm) and placed in a 37°C oven for 20 min. After crosslinking, scaffolds were removed from the molds. MB200, MB100, and NR samples were cut to a length of 15.0 mm and stored under nitrogen at 4°C. LB200 samples were placed in ddH₂O for 3 days to remove the NaCl porogen, dried, and stored under nitrogen at 4°C. All PPF composite materials were sterilized through exposure to UV light for 12 hours prior to scaffold fabrication. All subsequent material handling occurred under sterile conditions. The complete scaffolds were exposed to UV light for an additional 12 hours after fabrication.

2.5 Microparticle Solution Preparation

A solution of Pluronic F127 (PF127, 24 wt% in ddH₂O; BASF Corporation, Mount Olive, NJ) was made for use as a carrier for the PLGA microparticles for injection into the pores of the PPF scaffolds to fabricate LB200 samples. The use of a pluronic
solution was chosen for this study because of its unique property to behave as a liquid at low temperatures (0°C) and to form and remain as a gel at temperatures at and above room temperature. Prior to surgery, the cold (0°) PF127 solution was added to a vial containing PLGA microparticles (0.150 g microparticle/2 mL PF127 solution). The microparticle solution was kept on ice until just prior to implantation when it was shaken gently to obtain a homogeneous solution without creating air bubbles. The well-mixed suspension (75 µl) was taken up in a syringe and injected into the pores of the polymer scaffold.

2.6 Surgical Procedure

Ten healthy, female New Zealand white rabbits weighing between 2.5 and 3.5 kg were used in this study. Surgery was performed under general inhalation anesthesia. The anesthesia was induced by an intravenous injection of Hypnorm® (0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone) and atropine and maintained by a mixture of nitrous oxide, isoflurane, and oxygen through a constant volume ventilator. Antibiotic prophylaxis (Baytril 2.5%, Enrofloxacin, 5-10 mg/kg) was given to reduce the risk of peri-operative infection.

Animals were placed in a ventral position and immobilized on their abdomen for surgery. Hair from both legs of each animal was shaved and the skin was disinfected with povidine-iodine. A 4.5 cm longitudinal incision was made in the skin with a blade along the radius. Dissection of the muscle exposed the radius. The periosteum was removed and a 15.0 mm defect was created in the middle of the radius by a burr with a diamant blade with continuous saline cooling. The samples were inserted in the defect without external fixation. Finally, the muscle layers and then the skin were closed using
a 4-0 Vicryl sutures. All surgeries were conducted by the same investigator at the same
time.

A total of 20 scaffolds were implanted: 5 LB200, 5 MB200, 5 MB100, and 5 NR. A balanced split plot randomization scheme was used to locate the scaffolds so that the limb choice or formulation pairing would not affect the outcome [27]. At 12 weeks post-implantation, animals were euthanized with an overdose of Nembutal (pentobarbital). All implants and surrounding tissue were retrieved *en block*. Dutch and United States National Institutes of Health Guidelines for the Care and Use of Laboratory Animals were observed throughout the course of this study.

2.7 Light Microscopy (*histological scoring analysis and histomorphometric analysis*)

After retrieval, specimens were prepared for histological evaluation. Specimens were fixed in 4% formalin solution (pH = 7.4), dehydrated in a graded series of ethanol (from 70% (v/v) to 100%), and embedded in methylmethacrylate. After polymerization, samples were hemi-sectioned through the center of the defect area creating two separate samples of each implant. Subsequently, a minimum of three 10 μm thick sections were made using a modified microtome technique [28]. Sections were made axially through one implant sample and transversely through the second implant sample. Sections were stained with methylene blue and basic fuchsin to detect bone formation. Prepared sections where evaluated using light microscopy (Leica BV, Rijswijk, The Netherlands)

All histological sections were quantitatively assessed using a modified histological scoring analysis (Table 5-2) [29-33]. Samples were evaluated for: (1) bone growth into the PPF-composite scaffolds from the existing bone into the implant at the defect borders (bridging), (2) bone growth along the surface of the implant (guided-growth), (3) tissue response within the pores of the scaffold, and (4) degradation of the
polymer scaffold. Bone ingrowth and guided-growth were evaluated in the longitudinal sections only, while tissue response and polymer degradation were evaluated in both the longitudinal as well as the cross-sectional sections. Two blinded reviewers evaluated at least three sections for each scaffold. The reviewers reached a consensus on the score of each section and then assigned a final score to each scaffold. The scores of the scaffolds of each class were then averaged to determine the overall score for the class.

Further histomorphometric analysis of bone ingrowth into the various specimens was performed to determine the distance bone grew into the PPF scaffolds. Again, at least three histological sections were imaged and the edges of the defect border were determined and the distance of bone growth into the defect was measured using a Leica Qwin Pro image analysis system. The average of each scaffold was determined and then used to calculate the class average for n= 5 scaffolds.

2.8 Statistical Analysis

A one-way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparisons test was used to determine statistical significance with 95% confidence intervals (p<0.05) using GraphPad InStat version 3.05 statistical software (San Diego, California, USA). The results are reported as mean ± standard deviation for n=5

3. Results

3.1 Descriptive light microscopic evaluation

Gross analysis of the sections via light microscopy was first performed. Remaining PPF scaffolds could be easily identified as the polymer remained whitish even after specimen staining. For all implants, defect borders were easily identifiable.
Overall, scaffolds maintained their original shape, with the pores appearing square due to the use of NaCl crystals as the leachable porogen.

Analysis of longitudinal histological samples revealed that LB200 and NR samples showed bone ingrowth into the PPF/PLGA scaffolds at the defect borders, with much more bone present in the LB200 samples (Figure 5-2(a)). The in-growing bone was observed in close contact with the polymer implant, without an intervening fibrous tissue layer. Active bone formation within the pores was apparent, characterized by the presence of osteoblast-like cells and osteoid (Figures 5-2(b) and 5-3(c)). In addition to bone formation within the scaffold, LB200 samples achieved guidance of bone along the outer PPF surface (guided-growth) (Figures 5-2(a) and 5-2(d)). Further, a number of non-burst release samples showed cartilage formation at the outer side of the PPF implant (Figure 5-2(e)). At the ulna side of the implant, the ulnar bone appeared to have undergone extensive remodeling and hypertrophy. Fusion of the ulna and radius was apparent in all samples.

In locations where bone ingrowth was not observed, scaffold porosity was filled with mostly fibrous tissue and small blood vessels (Figures 5-3(a) and 5-3(b)). In MB200, MB100 and NR samples, PLGA microparticles were easily detected in the bulk of the polymer phase, appearing as circular structures (Figures 5-3(a) and 5-3(c)). Microparticles exposed to the surface of a pore created additional porosity filled with fibrous tissue. Inflammatory cells (giant cells and macrophages) were present within the scaffolds, but were usually associated with areas of micro-fragmentation of the scaffold polymer (Figures 5-3(c) and 5-2(d)). These areas were limited to the ends and the outside edges of the implants.

Review of the cross-sectional sections supported the findings of the longitudinal section analysis. More bone was found in sections taken closer to the defect edges (lower sections) than to the center of the defect (center sections). In addition, polymer
micro-fragmentation and inflammatory response cells were more prevalent in the lower sections.

Cross-sectional samples also provided information about the morphology of the guided bone growth over the LB200 samples. In several samples this bone displayed a structure similar to the original radius, i.e. with a medullary canal down the center filled with fatty tissue (Figure 5-3(e)). The guided-growth bone lacked such a medullary canal and was composed almost entirely of bone in remaining samples.

3.2 Quantitative Histological Analysis

Figure 5-4 shows the scoring results of bone bridging of the defect borders. Statistical analysis of these data showed no statistical differences ($p > 0.05$) between any of the scaffold formulations. Figure 5-5 provides data on the evaluation of the bone growth that occurred along the outside surface of the implant (guided-growth). Analysis of longitudinal sections showed that LB200 scaffolds exhibited significantly more guided bone growth than MB200, MB100, or NR scaffolds ($p < 0.05$). LB200 scaffolds averaged 80% of the defect length bridged with bone, while MB200, MB100 and NR averaged 10%, 5% and 0%, respectively. The results of the analysis of the tissue response within the pores of the PPF/PLGA composite scaffolds are given in Figure 5-6.

Evaluation of the longitudinal analysis suggests that, overall, LB200 samples included mostly bone and fibrous tissue, while MB200, MB100, and NR samples tended to possess more fibrous tissue with some inflammatory response. Nevertheless, no statistical difference was observed. Cross-sectional samples supported this trend, also suggesting a slight difference between the edge and the center sections. Results of polymer degradation analysis are presented in Figure 5-7. All sample formulations exhibited values close to 1, indicating minimal degradation of the polymer. Review of
the cross-sectional sections revealed that the degradation appeared to occur more
towards the ends of the scaffolds than the center (differences not significant).

Quantification of the depth of penetration of bone in scaffolds that exhibited
bridging of the defect borders can be seen in Table 5-3 and Figure 8. Great variations
occurred within all scaffold formulations, resulting in no statistically significant difference
between any of the formulations.

4. Discussion

The objective of this study was to examine \textit{in vivo} bone formation in response to
the controlled release of TP508 from PPF/PLGA composite scaffolds. We hypothesized
that variation in the release kinetics of TP508 would influence in the healing of a
segmental bone defect. More specifically, we hypothesized that a release profile with a
minimal initial burst release of TP508 would result in greater bone formation than the
same dose of TP508 released with a large initial burst. We supposed that, with a
reduction in the initial burst release, a lower dosage of TP508 would be required to
augment bone formation in segmental defects. Samples were implanted in a 15.0 mm
defect in the rabbit radius and assessed 12 weeks post-operatively using histological
and histomorphometric techniques. The results of this study indicate that our hypothesis
was incorrect, because the LB200 scaffolds showed a statistically greater amount of
bone formation than the other scaffold classes examined here.

The rabbit radius defect was selected as the model for bone defect repair for our
work as this model is widely used and accepted for the testing of both scaffold materials
and delivery of osteoinductive factors in bone sites [34]. One advantage of this model is
the lack of the need for external fixation of the bone due to the close proximity of the
ulna. On the other hand, the presence of the ulna complicates the healing response.
Ulnar hypertrophy and ingrowth into the defect area and implant were observed in our
samples and contributions from the ulna could not be excluded in our histological analysis.

Grading systems are commonly used for the quantification of histological data in a reproducible and comparative manner. In this study, a histological scale was used to assess bone ingrowth into the PPF/PLGA composite scaffolds, guided bone growth around the outside of the scaffolds, tissue response, and scaffold polymer degradation. Overall, bone ingrowth into the implanted scaffolds was limited. LB200 and NR samples exhibited penetration into 3/5 of the samples examined, with the amount and depth of penetration being greater in the LB200 samples than the NR samples (p<0.05). The biological influence of TP508 in orthopedic wound healing is not yet completely understood. It is currently believed that TP508 interacts with inflammatory cells causing an up-regulation of the cell's production of important growth and stimulating factors.

Substantial bone ingrowth early in the implantation period will lead to fixation of the implant at the defect site, offering a stable, splinted-like situation. This stabilized implant provided a rigid, osteoconductive surface for the guidance of bone over the polymer implant. This also explains the significant guided bone growth observed for three of the LB200 specimens, as these implants also displayed significantly more bone ingrowth at the defect edges.

Bone formation was limited in samples that exhibited a minimal burst release of TP508. For the minimum burst release samples, release of TP508 from the composite scaffold is due to the diffusion of the peptide out of the polymer matrix. As the peptide is embedded in PLGA microparticles that are entrapped in PPF, the peptide’s diffusivity is intrinsically linked to the crosslinking density of the PPF scaffold and its degradation rate.

Any polymer degradation evident within the PPF scaffolds occurred at the edges of the implants, and was limited to micro-fragmentation of the network. PPF degradation
has been shown to proceed through hydrolysis of its ester linkages. Although the degradation products, fumaric acid and propylene glycol, have been shown to be biocompatible, they are expected to elicit a mild inflammatory response due to the acidity of the fumaric acid [35]. In areas where micro-fragmentation occurred, inflammatory cells and macrophages were observed. In other areas of the scaffold, the polymer scaffold structure remained intact and the tissue response was mild with vascularization, collagen matrix production, and a limited amount of foreign body giant cells present. The tissue response in our implants was similar to the response seen in previous cranial implant studies [21, 36].

We have to notice that differences in material properties existed between the various implant classes. These differences were not expected to influence the osteoconductive capacity of the scaffolds. In order to incorporate the PLGA microparticles into LB200 samples, a Pluronic F127 solution was used. Pluronic F127 is a tri-block copolymer of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) that has been shown to exhibit low toxicity and has been explored for applications in drug delivery and tissue engineering [37, 38]. The effects of F127 on bone healing in critical sized defects in the rat calvarium were assessed by administering F127 either topically or systemically and in conjunction with demineralized bone powder, tricalcium phosphate, or non-grafted controls. Neither the presence of F127 nor the route of administration was shown to effect wound healing as measured by percent bone fill in histological sections [39]. Based on these results, we do not suppose that the inclusion of F127 altered the bone forming capacity of the LB200 class of scaffolds.

LB200 scaffolds also underwent leaching of its NaCl prior to implantation, while the remaining classes did not. Radiographs obtained 1-day post implantation showed that the radio-opaque salt was no longer present in the defect area (results not shown), indicating that the porogen was able to leach from the polymer leaving a porous scaffold
behind. The effects of a possible change in local salinity around the MB200, MB100, and NR implants are not known and could not be assessed in this study. Further studies are needed to investigate the effects of alterations in salinity on overall tissue response and bone formation.

5. Conclusions

This study sought to elucidate the effect of varied release kinetics from PPF/PLGA composite scaffolds on bone formation in 15.0 mm segmental defects in the rabbit radius. Altering the extent of the initial burst release of TP508 from the composite materials influenced the amount of bone formation, with a large burst release resulting in the greatest amount of bone formation. Our results suggest that the initial burst of TP508 led to bone growth at the borders of the defect and stabilization of the implant, increasing the osteogenicity of the scaffold surface over which bone could continue to grow.
<table>
<thead>
<tr>
<th>Scaffold Class</th>
<th>Release Profile</th>
<th>TP508 Dosage (µg)</th>
<th>Location of Microparticles</th>
<th>Pre-leaching</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB200</td>
<td>Large burst</td>
<td>200</td>
<td>Pores</td>
<td>Yes</td>
</tr>
<tr>
<td>MB200</td>
<td>Minimal burst</td>
<td>200</td>
<td>Polymer</td>
<td>No</td>
</tr>
<tr>
<td>MB100</td>
<td>Minimal burst</td>
<td>100</td>
<td>Polymer</td>
<td>No</td>
</tr>
<tr>
<td>NR</td>
<td>No release</td>
<td>0</td>
<td>Polymer</td>
<td>No</td>
</tr>
</tbody>
</table>
| TABLE 5-2  
<table>
<thead>
<tr>
<th>Histological Grading Scale</th>
</tr>
</thead>
</table>
| **Bridging of defect border**<sup>1</sup>  
Yes & 1  
No & 0  
**Guided bone growth (guided-growth)**<sup>1,2</sup>  
76 - 100% & 4  
51 - 75% & 3  
26 - 50% & 2  
1 - 25% & 1  
= 0% & 0  
**Tissue response within the pores of the polymer scaffold**  
Tissue in pores is mostly bone & 4  
Tissue in pores consists of bone with fibrous tissue and/or inflammatory response elements & 3  
Tissue in pores of polymer is mostly fibrous tissue, showing blood vessels and young fibroblasts invading the space with few macrophages present & 2  
Tissue in pores consists of giant cells and other inflammatory cells in abundance but connective tissue components in between & 1  
Tissue in pores is dense and exclusively of inflammatory type & 0  
**Polymer degradation**  
Decrease in polymer area & 3  
Excessive fragmentation (throughout polymer) & 2  
Minimal fragmentation (edges/outer-surface) & 1  
Intact polymer & 0  

<sup>1</sup> Bridging of the defect border and bone growth around the scaffold was assessed with longitudinal sections only.  
<sup>2</sup> Guided bone growth (guided-growth) is a measure of the distance the bone growth around the scaffold extends from the defect border towards the center of the defect.
<table>
<thead>
<tr>
<th>Scaffold Class</th>
<th>Ratio of Samples Exhibiting Bone Penetration</th>
<th>Distance of Penetration (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB200</td>
<td>3/5</td>
<td>0, 0, 1.2, 3.3, 7.5</td>
</tr>
<tr>
<td>MB200</td>
<td>2/5</td>
<td>0, 0, 0, 0.4, 0.8</td>
</tr>
<tr>
<td>MB100</td>
<td>1/5</td>
<td>0, 0, 0, 0, 2.4</td>
</tr>
<tr>
<td>NR</td>
<td>3/5</td>
<td>0, 0, 0.9, 1.3, 1.6</td>
</tr>
</tbody>
</table>
Figure 5-1: Cumulative mass release of TP508 from PPF composite scaffolds into PBS at 37°C for scaffold classes examined in vivo. Results represent mean ± standard deviation for n=3. Adapted from [20].
Figure 5-2: Histological sections of PPF/PLGA scaffolds. (a) Bone ingrowth into and guidance around PPF/PLGA scaffold (class LB200); (b,c) pore bone fill, active bone formation, bone/implant contact (class LB200); (d) scaffolds with no bone ingrowth exhibiting bone growth around scaffold (guided growth; class MB200); (e) cartilage formation (class MB200).
Figure 5-3: Histological sections of PPF/PLGA scaffolds. (a, b) Fibrous tissue, collagen fibers, blood vessels (classes NR and LB200); (c, d) polymer fragmentation with increased inflammatory response (classes MB100 and MB200); (e) cross-sectional section with no ingrowth but extensive growth around scaffold (guided growth; class LB200).
Figure 5-4: Results of histological scoring of longitudinal sections for bone growth into defect at defect borders (bridging). Results are means ± standard deviation for n=5.
Figure 5-5: Results of histological scoring of longitudinal sections for bone growth around the outside of the implant (guided-growth). Results are means ± standard deviation for n=5.
Figure 5-6: Results of histological scoring for tissue response within the pores of the implant. (A) results of analysis of longitudinal sections; (B) results of analysis of lower cross-sectional sections (■) and upper cross-sectional sections (□). Results are means ± standard deviation for n=5.
Figure 5-7: Results of histological scoring for polymer degradation. (a) Results of analysis of longitudinal sections; (b) results of analysis of lower cross-sectional sections (■) and upper cross-sectional sections (□). Results are means ± standard deviation for n=5.
**Figure 5-8**: Average maximum distance of bone penetration into the polymer scaffold for each scaffold class. Results are means ± standard deviation for n=5.
CHAPTER VI

A Comparative Analysis of Radiography, Micro-Computed Tomography, and Histology for Bone Tissue Engineering*

Abstract

This study focused on the assessment of radiography, micro-computed tomography, and histology for the evaluation of bone formation in a 15.0 mm defect in the rabbit radius after the implantation of a tissue engineered construct. Radiography was found to be useful as a non-invasive method of obtaining images of calcified tissue throughout the time course of the experiment. With this method, however, image quality was low making it difficult to obtain precise information on the location and quantity of the bone formed. Micro-computed tomography was utilized to create three-dimensional reconstructions of the bone (25 μm resolution). These reconstructions allowed for greater special resolution than the radiography, but did not allow for imaging of the implanted scaffold material or the surrounding, non-mineralized tissue. To visualize all materials within the defect area at the cellular level, histology was used. Histological analysis, however, is a destructive technique which did not allow for any further analysis of the samples. Each technique examined here has its own advantages and limitations, but each yields unique information regarding bone regeneration. It is only through the use of all three techniques that complete characterization of the bone growth, tissue and construct response after implantation in vivo can be obtained.

1. Introduction

Over the past decade, researchers in the area of tissue engineering have made significant progress towards the regeneration of natural, functional tissue within bone defect sites. A large number of materials have been identified as potential candidates for use as scaffolds for cellular ingrowth and/or as carriers of osteoinductive entities such as osteogenic cell populations, growth factors and cytokines. Initial studies to evaluate the scaffold characteristics often occur in vitro. In vivo studies must also be conducted, however, for a complete understanding of the material and its ability to assist in bone formation. Unfortunately, in vivo studies are typically more expensive and time consuming than in vitro studies. Additionally, sample numbers are limited due to ethical considerations in the use of living animals. It is imperative, therefore, that proper analytical techniques are chosen in the in vivo studies in order to obtain the most accurate and complete information as possible.

Radiographic and histological techniques are the traditional methods employed for the assessment of bone growth into defect sites provided with tissue engineering constructs. As radiographs are non-destructive, they are valuable for following the formation of bone within a defect site over time using standardized serial radiographs obtained on a given sample. Nevertheless, radiographs are two-dimensional representations of three-dimensional structures and their interpretation can be difficult. Besides, the spatial resolution of conventional radiography is very low, which excludes a detailed analysis of tissue structures. Histological techniques involve the analysis of two-dimensional slices prepared from tissue samples. Through the use of proper staining techniques important features such as bone, fibrous tissue, blood vessels and cells can be located. Measuring histomorphometric parameters such as percent bone fill and bone area coverage can yield quantitative results. By analyzing various histological
sections of the same tissue sample, these results are often extrapolated to the three-dimensional structure. This extrapolation, however, can result in incorrect three-dimensional reconstructions due to the anisotropic nature of bone.

A newly developed alternative to plane radiographs and histology is micro-computed tomography (μCT). μCT is a non-destructive technique that is easy to perform. Using a fine-beam x-ray source and a two-dimensional detector array, multiple images are taken for consecutive two-dimensional horizontal slices as the sample slowly rotates. Computer algorithms are then used to reconstruct the sample’s internal and external structure from the many two-dimensional images collected. Quantitative results can also be obtained including bone volume and surface area to volume ratios. μCTs employing conventional x-ray sources have a resolution down to 10 μm, while equipment using synchrotron radiation have a resolution down to 1 μm [1-3]. Difference in attenuation coefficients between bone, fibrous tissue, and polymeric implants allow for reconstruction of bone independent of the surrounding materials [4].

The aim of the current study is to compare the various available analytical techniques for the assessment of bone ingrowth into defects treated with tissue-engineered constructs and to determine their advantages as well as their disadvantages. To perform this analysis, poly(propylene fumarate)-based scaffolds carrying the osteogenic peptide TP508 (Chrysalin®, MW = 2,311; AGYKPDEGKRGDAEGDSGFPV) with different release profiles and dosages were implanted in 15.0 mm defects in the rabbit radius. The classes employed in this study included: (a) scaffolds loaded with 200 μg TP508 exhibiting a large burst release (LB200); (b) scaffolds loaded with 100 μg TP508 exhibiting a minimal burst release (MB200); (c) scaffolds loaded with 100 μg TP508 exhibiting a minimal burst release (MB100); and, (d) blank scaffolds without TP508 (NR). Individual samples were followed
through radiography, micro-computed tomography and histology, allowing for comparison of the different techniques with each sample.

2. Materials and Methods

2.1 Methods

Synthesis of PPF and the crosslinking agent poly(propylene fumarate) (PPF-DA), fabrication of the TP508 loaded PLGA microparticles and PPF/PLGA composite scaffolds, and surgical procedures were performed as previously described [5]. Dutch and United States National Institutes of Health Guidelines for the Care and Use of Laboratory Animals were observed throughout the course of this study.

2.2 Experimental Design

A total of 20 scaffolds were implanted in 15.0 mm defects in the rabbit radius: 5 LB200, 5 MB200, 5 MB100, and 5 NR. A balanced split plot randomization scheme was used to locate the scaffolds so that the limb choice or formulation pairing would not affect the research outcome [6].

2.3 Radiography

Under anesthesia, each rabbit was positioned prone with the forelimbs externally rotated. Serial radiographs of both forelimbs were made at 0, 2 and 12 weeks postoperatively on a Siemens Mobilett X-ray Machine.

Radiography obtained at 12 weeks were evaluated for defect bridging and bone formation by two clinicians who were blinded to the implant type using a modified scoring system (Figure 6-1) [7-10]. As little to no bone formation was observed in the 0 and 2
week radiographs, these time points were used as negative controls to assist in the location of the original defect site in the radiographs obtained at the later time points.

Based on the radiographic assessment, ten scaffolds were selected for further evaluation with both μCT and histology. The selected samples included five LB200 samples (assigned as Group A) and 2 MB200, 2 MB100, and 1 NR samples (assigned as group B). The choice was based on the amount of bone ingrowth in the defect space, where Group A samples displayed significantly more bone ingrowth than Group B samples (minimal ingrowth).

2.4 Sample Harvest

At 12 weeks post-implantation, all animals were euthanized and the defect area and surrounding tissues was retrieved. Following retrieval, samples including surrounding tissues were fixed in a 10% formalin solution. After fixation, specimens were placed in PBS and stored at 4°C until further analysis.

2.5 Micro-Computed Tomography

For one run of the μCT (ScanCo μCT-80, ScanCo, Basserdorf, Switzerland), five specimens were fixed vertically within the sample holder, covered with PBS, and placed in the μCT sample holder. Specimens were scanned at an energy of 50 kV and an intensity of 80 μA, with a nominal resolution of 25 μm. A long integration time (300 ms) was used in order to reduce the signal-to-noise ration in the presence of the surrounding tissue and PBS.

Radiographs and gross analysis of the specimens were used to locate the defect borders. Scanning of the samples began just above the upper defect border and continued for 20 mm in order to ensure inclusion of the entire defect area. The full 3-D
reconstructions and 12-week radiographs were then used to determine the location of
the defect area more precisely. Next, the ulna and radius between the defect edges
were isolated and reconstructed to obtain the ulna/radius complex. Finally, the radius
was isolated from the ulna/radius complex and reconstructed independently.

2.6 Histology

After analysis by μCT, specimens were dehydrated in a graded series of ethanol
(from 70% (v/v) to 100%), and embedded in methylmethacrylate. After polymerization,
additional radiographs were taken of each sample to assist in location of the defect
borders. Subsequently, samples were hemi-sectioned through the center of the defect
area creating two separate samples for each implant. A series of 10-μm thick sections
were made axially through one implant sample and transversely through the second
implant sample [11]. Sections were stained with methylene blue/basic fuchsin and
examined using light microscopy (Leica BV, Rijswijk, The Netherlands). A minimum of
three sections per sample were evaluated for the presence of guided bone formation
along the outer surface of the polymer scaffold using cross-sectional sections and bone
bridging of the defect borders and the extent of bone formation outside the polymer
scaffold using longitudinal samples. Two observers who were blinded to the implant
type conducted the evaluations using a modified scoring system (Table 6-1). The
reviewers reached a consensus on the score of each section and then assigned a final
score to each scaffold. The scores of the scaffolds of each class were then averaged to
determine the overall score for the class.

Further histomorphometric analysis of bone ingrowth into the various specimens
was performed to determine the distance bone grew into the PPF scaffolds. Again, at
least three histological sections were imaged and, using a Leica Qwin Pro image
analysis system, the edges of the defect border were determined and the distance of bone growth into the defect was measured.

**Statistical Analysis**

For analysis of full radiographic data, a one-way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparisons test was used to determine statistical significance with 95% confidence intervals (p<0.05) using GraphPad InStat version 3.05 statistical software (San Diego, California, USA). For comparison of methods, a two-tailed t-test was used. All results are reported as means±standard deviation for n=5.

3. Results

3.1 Complete radiographic analysis

Radiographic analysis was performed as a noninvasive means to assess bone formation within the defect sites. Radiographs were taken just after surgery and at 2 and 12 weeks. Representative radiographs from 12 weeks postoperatively from each scaffold class are depicted in Figure 6-2. The polymeric scaffolds are not visible due to the low x-ray attenuation coefficient of polymers. As bone formation was not apparent in any of the samples at 2 weeks, radiographic scoring was performed on the 12-week images only. Blinded radiographic evaluation of bone area coverage and bone union shows that implants with a large initial release of TP508 (LB200) obtain higher scores (p < 0.05) for both parameters than implants with extended release (MB200 and MB100) or no release (NR) (Figure 6-3).
3.2 Comparative Analysis using Radiography, Micro-Computed tomography and Histology

3.2.1 Group A - Descriptive

Radiographs from a representative Group A sample are depicted in Figure 6-4. In this sample, the two-dimensional radiographs suggest full bridging of the defect as well as almost complete bone fill within the defect area. Varying densities of the radiograph indicate differing amounts of mineralization within the defect area, with a greater amount of mineralization occurring along the outside of the defect area than in the center of the defect area.

Micro-computed tomography was used to create three-dimensional images of the defect area as well as to determine the quantity of bone formed. Images of mineralized bone excluding surrounding tissue and polymer implant appeared easy to obtain, as the attenuation coefficient for bone is much greater than that of the other materials. Representative reconstructions for the radius and ulna between the defect edges from the same sample can be found in Figure 6-5. The comparative analysis of the μCT versus the radiography can best be explained with help of the μCT image shown in Figure 6-5, which is of the same specimen as the radiograph presented in Figure 6-4. This radiograph suggests that bone is spanning from one defect border to the other, covering almost the entire defect area. This observation corresponds very well with the μCT picture. In addition, the gap in bone between the radius and ulna seen in the radiograph is also evident in this μCT image. On the other hand, when the sample is rotated 180°, it is apparent that bone formation was not uniform within the defect area (Figure 6-5b). From this angle, large areas without bone can be observed. The shape of the empty area suggests the location of the polymer scaffold, but cannot be verified
with this technique. µCT also allows the creation of longitudinal as well as cross-sectional planar sections of the complete sample (Figures 6-5d and 6-5e). Evidently, bone growth along the outside edge of the cylindrical implant can be seen. The newly formed bone, although not exactly located where the original radius had been, has a similar macroscopic structure as the original radius, with a dense exterior and the formation of a central canal. Finally, ulnar hypertrophy can also be observed in the cross-sectional images. Comparison of all available µCT images with the radiographs showed similar observations.

Lastly, 10-µm thick slices were made from the samples, for analysis using histological techniques. In these sections, remaining PPF polymer can easily be detected as the polymer remains unstained and appears white under light microscopy. Cross-sectional sections (Figures 6-6a and 6-6b) allowed for localization and characterization of the bone growth outside the scaffold as these sections allowed for complete visualization of the circumferential perimeter of the implant. Longitudinal sections revealed that bone ingrowth into the scaffold from the edge of the guided-growth was limited (Figures 6-6c and 6-6d). Further, these longitudinal sections allowed analysis of the defect border and the extent of bone penetration into the scaffold could be measured. All images in Figure 6-6 were histological sections of the same specimen as shown in radiograph of Figure 6-4 and the µCT image of Figure 6-5. Under the condition that the proper direction of sectioning is chosen (cross-sectional versus longitudinal), similar information can be obtained with histology compared to µCT.

3.2.2 Group B - Descriptive

Radiographs, µCT and histology for a representative sample from Group B can be found in Figure 6-7. All techniques show minimal bone growth in the defect area.
However, only evaluation of the histological sections provides information on the remaining polymer as well as the tissue and cell populations within the pores of the scaffold.

3.2.3 Groups A and B - Quantitative

Quantitative data of all specimens using radiography, μCT as well as histology are summarized in Table 6-2. Samples were assigned to groups according to their radiographic evaluation. Group A samples showed more bone formation and defect bridging than the samples of Group B. Results from the μCT measurements support this finding with more bone volume per unit length in the Group A samples than the Group B samples.

Samples A1, A2, A4, and A5 had radiographic scores of 4 for both parameters examined, indicating complete closure of the defect. In contrast, results from μCT and histology both show that complete closure of the defect area was not obtained. It is only through the application of these additional techniques that a clear understanding of the location and structure of the regenerated tissue is obtained. On the other hand, results from radiography, μCT and histology all yielded the same results in samples with minimal bone formation.

4. Discussion

In vivo experiments are a necessary step in the characterization of potential materials for bone tissue engineering applications. These studies can be costly and time consuming, so each experiment must be carefully planned and executed in order to maximize the amount of information gathered from each one. In the present work, three analytical techniques currently available for the assessment of bone structure were
utilized for evaluation of bone ingrowth in segmental radius defects after the implantation of a biodegradable polymer scaffold that released osteogenic peptides at varied release rates and dosages.

To allow a proper comparison and in order to decrease the number of samples analyzed with all three techniques, ten samples were selected. Five of those specimens showed radiographically complete bone filling of the defect area after 12 weeks of implantation, while the five other specimens showed no bone filling at all after the same time period.

Our results and comparison show that each method explored in this study, radiography, micro-computed tomography, and histology, can be used to gather unique information regarding tissue engineering scaffolds and their ability to assist in the regeneration of bone tissue. Radiographs are very useful as a non-invasive method for the imaging of tissues with high x-ray attenuation coefficients such as bone. Within less than an hour an image of gross structure of the bone inside a living animal can be obtained. The animal can be photographed repeatedly, allowing for the visualization of a defect area multiple times over the course of the implantation period. Polymers do not usually appear in radiographs as they have very low x-ray attenuation coefficients [12]. This can be an advantage as it allows for an unobstructed view of tissue growth into the defect area. Unfortunately, it does not allow for visual localization of the implant within the defect site, which can be useful in situations where movement or prolonged maintenance of the implant is a potential problem. Additives such as barium sulfate, supplemented to the polymer prior to fabrication of the scaffold can solve this visualization problem. Although, it has to be noted that such additives may interfere with the tissue response [13-15]. Metals and ceramics have higher attenuation coefficients and can be easily recognized in radiographic pictures [10, 16].
Interpretation of x-ray images is not necessarily a simple process. Therefore, evaluation of radiographs has always to be done by multiple experienced evaluators. Each point on a plane radiographic image is a function of the attenuation coefficients of all materials through which the x-ray beam passes. This results in the mapping of a three-dimensional object onto a two-dimensional image. This loss of the three-dimensional structure results in a loss of depth information, making it impossible to determine the precise location of a feature [12]. Consequently, guided bone growth around the scaffold will always be difficult to discern from bone in-growth into the tissue-engineered construct.

Micro-computed tomography is a relatively new, non-destructive technique for imaging bone in three dimensions. As with plane radiographs, x-rays are directed at a sample and a detector on the far side of the object detects the emitted rays to create a two-dimensional picture. With \( \mu \text{CT} \), however, this process is repeated multiple times as the sample rotates 360° at each two-dimensional vertical level of the sample. This method produces hundreds of two-dimensional images from which a three-dimensional image can be extracted using appropriate computer analysis software.

Without the need for any specimen preparation, \( \mu \text{CT} \) was found to produce good-quality topographical images of our samples that can be rotated and viewed from any angle. In all our images, bone was easily distinguishable from not-bone (polymer and soft tissue) due to the great differences in their x-ray attenuation coefficients. An additional advantage is that non-destructive sectioning of the sample can be done in all directions for visualization of the bone structure within the defect site. The use of \( \mu \text{CT} \) also allows for quantitative measurements of many parameters important of the characterization of bone. For all types of bone samples, bone volume can be measured directly. For trabecular samples, important indices such as surface area, trabecular
thickness, trabecular separation, and trabecular number can also be determined directly [4]. As with plane radiographs, however, visualization of a polymer is not possible [4]. In addition, the resolution of μCT is at the supracellular level and cannot be used to obtain detailed information about the cellular bone healing response. Even if and when the resolution of micro-computed tomography reaches the cellular level, the low x-ray attenuation coefficient of the cells will still render visualization impossible [4].

Histological analysis is commonly employed for analysis of bone structure after retrieval of a specimen. With this technique, samples are embedded in methylmethacrylate and cut into micron-thick sections. Sections can be stained for visualization of specific features such as bone, fibrous tissue, blood vessels and cells. Qualitative analysis is obtained through viewing the sections under a light microscope. Unlike with radiographs and μCT reconstructions, histology allows for visualization and characterization of the microscopic nature of the tissue in and around an implant. Resolution is such that individual cells can be seen in a histological section.

Quantitative results can be obtained through the use of computer-based histomorphometric measurements. Here again, two-dimensional representations of a three-dimensional object must be employed. To overcome this limitation, serial sections can be obtained and 3-D reconstructions can be made with the assistance of stereomicroscopy. Loss of large quantities of tissue (up to hundreds of microns) is not uncommon during sectioning. The reconstructions, therefore, are just estimations of the real structure of the sample. Histological techniques certainly offer a better representation of the three-dimensional structure of bone tissue than plane radiographs. They are, however, much more labor intensive and time consuming. Additionally, histology is a destructive technique that does not allow for further testing of a given sample or for corrections when for example the wrong direction of sectioning is chosen.
5. Conclusions

Radiography, micro-computed tomography, and histology all have associated advantages and limitations. Each technique, however, gives important information regarding the *in vivo* response to the implantation of tissue engineering constructs. Used together, they work in cooperation to yield the most complete understanding of tissue formation and regeneration.
<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bridging of defect border</td>
<td></td>
</tr>
<tr>
<td>(longitudinal sections)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td>Presence of guided bone growth</td>
<td></td>
</tr>
<tr>
<td>(cross-sectional sections)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td>Extent of guided bone growth</td>
<td></td>
</tr>
<tr>
<td>(longitudinal sections)</td>
<td></td>
</tr>
<tr>
<td>74-100%</td>
<td>4</td>
</tr>
<tr>
<td>50-74%</td>
<td>3</td>
</tr>
<tr>
<td>25-49%</td>
<td>2</td>
</tr>
<tr>
<td>1-24%</td>
<td>1</td>
</tr>
<tr>
<td>0%</td>
<td>0</td>
</tr>
</tbody>
</table>

1 Guided bone growth is a measure of the distance the guided bone growth extends from the defect border towards the center of the defect.
<table>
<thead>
<tr>
<th>Table 6-2: Quantitative results of all samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>A1  A2  A3  A4  A5  Mean Group A  B1  B2  B3  B4  B5  Mean Group B</td>
</tr>
<tr>
<td>---------------------------------------------</td>
</tr>
<tr>
<td><strong>Radiography Scoring</strong></td>
</tr>
<tr>
<td>Fill</td>
</tr>
<tr>
<td>Union</td>
</tr>
<tr>
<td><strong>MicroCT</strong></td>
</tr>
<tr>
<td>Bone volume per mm (mm³/mm)</td>
</tr>
<tr>
<td><strong>Histology Scoring</strong></td>
</tr>
<tr>
<td>Bridging</td>
</tr>
<tr>
<td>Guided bone growth (cross-sectional)</td>
</tr>
<tr>
<td>Guided bone growth (longitudinal)</td>
</tr>
<tr>
<td>Penetration distance (mm)</td>
</tr>
</tbody>
</table>

1Histology scoring scales for bridging, guided bone growth can be found in Table 6-1
* Statistical difference between Group A and Group B, p < 0.01
** Statistical difference between Group A and Group B, p < 0.05
**Figure 6-1:** Schematics used to guide investigators in the quantitative evaluation of the radiographs. (a) Radiographic evaluation of the degree of union. Percentage values refer to the percent of the given defect border that is filled with bone that spans the defect. (b) Radiographic evaluation of the bone formation in the defect. Percentage values refer to the percent of the defect area that is filled with bone.
Figure 6-2: Representative radiographs from each scaffold classes taken 12 weeks post-operatively. (a) LB200, (b) MB200, (c) MB100, and (d) NR.
Figure 6-3: Results from radiographic scoring for the amount of bone formation within the defect area (a) and, the amount of bridging of one defect end with the other (b). Results represent mean±standard deviation for n = 5.
Figure 6-4: Radiographs of representative sample for Group A. Radiograph in (a) was taken prior to sacrifice at 12 weeks post-operatively; radiograph in (b) was taken after embedding of the sample in methylmethacrylate.
Figure 6-5: μCT reconstructions of representative sample for Group A; (a) position 1; (b) rotated 180°; (c) longitudinal section; cross-sectional section.
Figure 6-6: Histological sections from representative sample from Group A; (a, b) cross-sectional sections; (b, c) longitudinal sections of the implant. Bone overgrowth can be observed on the top of the implant samples in c and d.
Figure 6-7: Radiography (a), μCT (b), and histology (c,d) images from representative sample from Group B.
CHAPTER VII

In Vivo Degradation of Porous Poly(propylene Fumarate)/(Poly(DL-Lactic-co-Glycolic Acid) Composite Scaffolds*

Abstract

This study investigates the in vivo degradation of poly(propylene fumarate) (PPF)/poly(DL-lactic-co-glycolic acid) (PLGA) composite scaffolds designed for controlled release of osteogenic factors. PPF/PLGA composites were implanted into 15.0 mm segmental defects in the rabbit radius, harvested after 12 and 18 weeks, and analyzed using histological techniques to assess the extent of polymer degradation as well as the tissue response within the pores of the scaffolds. Polymer degradation was limited to micro-fragmentation of the scaffold at the ends and edges of the implant at both 12 and 18 weeks. The tissue within the pores of the scaffold consisted of fibrous tissue, blood vessels and some inflammatory cells. In areas where polymer breakdown was evident, an increased inflammatory response was observed. In contrast, areas of bone ingrowth into the polymer scaffold were characterized by minimal inflammatory response and polymer degradation. Our results show that minimal degradation of porous PPF occurs within 18 weeks of implantation in a rabbit model. Further, the in vivo degradation data of porous PPF/PLGA scaffolds are very comparable with earlier obtained in vitro data.

1. Introduction

When a bone defect arises that the body cannot heal through its normal wound healing response, surgical intervention is required. Currently, autografts, allografts, and

* This paper has been submitted for publication as: E.L. Hedberg, H.C. Kroese-Deutman, C.K. Shih, R.S. Crowther, D.H. Carney, A.G. Mikos, J.A. Jansen, "In vivo degradation of porous poly(propylene fumarate)/(poly(DL-lactic-co-glycolic acid) composite scaffolds," Biomaterials.
non-degradable bone cements are commonly employed to replace the missing tissue. All of these treatments, however, have associated limitations such as donor site morbidity, pathogen transfer, and complications due to differences in the mechanical properties of the implant and the surrounding bone [1]. In an attempt to overcome these difficulties, researches are focusing on the induction of tissue regeneration through the implantation of biodegradable scaffolds that provide temporary support for the migration, proliferation, differentiation, and eventual extra-cellular matrix production of bone forming cells.

Poly(propylene fumarate) (PPF) is a synthetic polymer currently being explored for use in bone tissue engineering [2-9]. PPF is a linear polyester that contains multiple unsaturated double bonds that are available for covalent crosslinking of the polymer in the presence of free-radical initiators. Crosslinked PPF has been shown to be biocompatible [2, 5, 8] and support bone formation both in vitro [9] and in vivo [6-8]. Although useful for the guidance of bone formation within a defect area, PPF scaffolds alone do not induce bone formation [2, 5]. In large defects where induction is necessary, osteogenic proteins or peptides can be incorporated and released from the PPF scaffolds.

To create PPF-based controlled release scaffolds, poly(DL-lactic-co-glycolic acid) (PLGA) microparticles containing the desired factor can be added to the polymer solution prior to crosslinking. Initial characterization of these PPF/PLGA composites focused on the release kinetics of the osteogenic peptide TP508 (Chrysalin®, MW = 22,311; AGYKDEGKRGDACEGDSGGPFV) [3] as well as the rate of degradation of the composites in vitro [10]. These studies were performed in controlled environments in vitro to allow for screening of multiple scaffold formulations as well as reasonable comparison with other similarly tested materials.
More recently, an *in vivo* experiment examining the effect of varied release kinetics and dosages of TP508 from PPF/PLGA composite scaffolds on bone formation after 12 weeks in a segmental defect in the rabbit radius was conducted [6]. Four scaffold classes were employed: (1) a large burst release scaffold loaded with 200 μg TP508 (LB200); (2) a minimal burst release scaffold with loaded with 200 μg TP508 (MB200); (3) a minimal burst release scaffold loaded with 100 μg TP508 (MB100); and (4) no release of TP508 (NR). Results demonstrated that LB200 samples exhibited the greatest amount of bone formation after 12 weeks and that the formation was characterized by guided growth along the surface of the polymer scaffold.

The objective of the study presented here is to assess the effects of the varied release kinetics and dosages of TP508 from PPF/PLGA composite scaffolds on polymer degradation *in vivo*. Scaffold formulations as described above were implanted in 15.0 mm defects in the rabbit radius. At 12 and 18 weeks post-operative, samples were retrieved and polymer degradation and tissue response within the pores of the scaffolds were assessed using histological techniques.

2. Materials and Methods

2.1 Methods

Synthesis of PPF and the crosslinking agent poly(propylene fumarate) (PPF-DA), fabrication of the TP508 loaded PLGA microparticles and PPF/PLGA composite scaffolds, and surgical procedures were performed in the manners previously described [6]. Dutch and United States National Institutes of Health Guidelines for the Care and Use of Laboratory Animals were observed throughout the course of this study.
2.2 Experimental design

A total of 40 scaffolds were implanted in 15.0 mm defects in the rabbit radius: 10 LB200, 10 MB200, 10 MB100, and 10 NR (Table 7-1). A balanced split plot randomization scheme was used to locate the scaffolds so that the limb choice or formulation pairing would not affect the research outcome [11]. At 12 and 18 weeks post-implantation, 5 animals from each scaffold class were euthanized and defect area including surrounding tissues was retrieved.

2.3 Sample Evaluation

After retrieval, specimens were prepared for histological evaluation. Specimens were fixed in 4% formalin solution (pH = 7.4), dehydrated in a graded series of ethanol (from 70% (v/v) to 100%), and embedded in methylmethacrylate. After polymerization, a minimum of three 10 μm thick sections were made cross-sectionally through the ulna and radius at the center of the defect area using a modified microtome technique [12]. Sections were stained with methylene blue (stains cells blue)/basic fuchsin (stains bone red) and examined using light microscopy (Leica BV, Rijswijk, The Netherlands).

All histological sections were quantitatively assessed using a modified histological scoring analysis (Table 7-2) [6]. Samples were evaluated for: (1) degradation of the polymer scaffold, and (2) tissue response within the pores of the scaffold. At least three sections were evaluated for each scaffold by two reviewers blinded to the scaffold classes. The reviewers reached a consensus on the score of each section and then assigned a final score to each scaffold. The scores of the scaffolds of each class were then averaged to determine the overall score for the class.
Statistical Analysis

Single factor non-parametric analysis of variance (ANOVA) (Kruskal-Wallis Test) followed by a Dunn’s multiple comparison test was used to determine statistical significance using GraphPad InStat version 3.05 statistical software (San Diego, California, USA). The results are reported as means±standard deviation for n=5.

3. Results

3.1 Descriptive Light Microscopy

Gross analysis of the sections via light microscopy was performed. Remaining PPF scaffolds could be easily identified, as the polymer remained whitish even after specimen staining. For all implants, defect borders were easily identifiable. Overall, scaffolds maintained their original shape, with the pores appearing square due to the use of NaCl crystals as the leachable porogen.

For all scaffold formulations, observable degradation of PPF/PLGA composite scaffolds was characterized by micro-fragmentation of the scaffold network (Figures 7-1a and 7-1b) and the presence of a large number of inflammatory response cells including multinucleated giant cells (Figure 7-1c). Degradation of the PLGA microparticles within the intact PPF network was evident through the presence of circular structures filled with tissue connected to the porous network of the scaffold (Figure 7-1d). The degradation of the PLGA did not elicit an increased inflammatory response in the immediate area of the microparticle. At both time points, micro-fragmentation of the scaffolds was localized at the defect borders as well as the outside surface of the implant. No effect of TP508 release kinetics or dosage on polymer degradation was observed.
Independent of scaffold formulation, bone formation was observed in the pores of the samples both on the side closest to the ulna (Figures 7-2a) and the side closest to the external edge of the scaffold (Figures 7-2a through 7-2c). Active bone formation was characterized by the presence of osteoblasts within the pores (Figure 7-2d). Bone formation could not be associated with degradation of the PPF scaffold. Additionally, a small number of samples exhibited areas of cartilage formation near the interface between the ulna and PPF implant (Figure 7-2e).

3.2 Histological Scoring

Histological sections from both 12 and 18 weeks were scored by two observers blinded to the scaffold classes for both polymer degradation and tissue response within the scaffold. Results of these analyses can be seen in Figure 7-3. Polymer degradation scores show that degradation was limited for all polymer classes at both time points, with no statistical differences obtained. An increase in the standard deviations is observed, indicating an increase in the range of scores within a given scaffold class. The tissue response within the pores was characterized mainly by the presence of fibrous tissue and blood vessels. Frequently LB200 samples exhibited increased bone formation in the pores of the scaffold, although no statistical difference was found between scaffolds of this class and scaffolds of the remaining classes.

4. Discussion

The objective of the current study was to assess the effect of varied release kinetics and dosages of TP508 from PPF/PLGA composite scaffolds on the extent of degradation and the accompanying tissue response within the pores of the composite scaffolds. A previous study has reported on the bone response and overall tissue response 12 weeks after implantation of the scaffold classes examined here. Results of
this study showed that bone growth was characterized by limited bone bridging of the
defect border and guided bone growth along the surface of the polymer implant. As the
focus of the current study was the degradation of the polymer scaffolds and the
associated tissue response within the scaffold, guided bone growth was not further
assessed here.

PPF is a synthetic linear polyester being explored for bone tissue engineering
applications. Initial studies have shown that PPF scaffolds possess many of the desired
characteristics for use as a biodegradable scaffold including biocompatibility, [2, 6, 13]
mechanical integrity, [10, 14] and biodegradability [15]. In order for a tissue engineering
implant to be successful over the long term, the implant material must have a rate of
degradation that acts in concert with the ingrowth of new bone. Ideally the scaffold
would degrade slowly enough to maintain structural support during the initial stages of
bone formation, but fast enough to allow space for continuous growth of new bone.

Numerous degradation studies of PPF scaffolds have been conducted examining
different scaffold formulations and timescales [10, 14, 16-18]. As with many
biocompatible polyesters, PPF undergoes bulk degradation through hydrolysis of the
ester linkages [19]. This form of degradation allows for maintenance of the scaffold
structure while the random chain scission at the ester linkages slowly decreases the
degree of crosslinking within the network, resulting in a decrease in mechanical
properties [20]. After some time, the extent of crosslinking is decreased enough to allow
for the outward diffusion of degradation products, resulting in a loss of mass and
structural integrity of the scaffold. In vitro studies conducted by Timmer et al.
demonstrate that solid networks of PPF crosslinked with the crosslinking agent PPF-DA
and the photoinitiator bis(2,4,6-trimethylbenzoyl) phenylphosphine oxide (BAPO) exhibit
an initial increase in mechanical properties during the first 6 weeks in PBS at a pH of 7.4
[14]. After this period, the mechanical properties decrease slightly over the course of 52
weeks, but do not decrease below their initial values. Additionally, changes in length and diameter were minimal over the same time period [14]. Solid samples, however, are expected to degrade faster than porous networks as there is less exchange of fluid within the network and a buildup of acidic degradation products trapped within the network that leads to auto-catalyzed degradation [21]. Porous scaffolds crosslinked with BAPO without the presence of PPF-DA have been examined in a similar study conducted by Fisher et al. and been shown to maintain their integrity through 26 weeks in vitro [18]. BAPO, however, is a much more effective crosslinking system than BP, and its use results in a greater degree of crosslinking in PPF/PPF-DA samples [22]. With fewer crosslinks, few chain scissions are needed to degrade the PPF scaffold into small enough pieces to be removed for the defect site. Therefore a recent in vitro study involving porous PPF/PPF-DA scaffolds crosslinked with BP showed that the scaffolds maintain mechanical properties and polymer mass through 26 weeks. This study also examined the effects of the addition of PLGA microparticles into the polymeric phase of the PPF scaffolds and found that the addition of the microparticles does not alter the rate of degradation of the composite scaffolds compared to unloaded PPF scaffolds over the same time period [10].

However, the in vivo environment is much more complex and difficult to predict than the in vitro setting, particularly with acellular in vitro experiments. Implant size and location, health of the animal, and enzymatic and local cellular activity are all factors that can influence the rate of degradation of a given polymer scaffold [23, 24]. Accordingly, in general, correlations between in vitro and in vivo results can be difficult to make. Nevertheless, in this study the in vivo degradation of the PPF/PLGA composite scaffolds appears similar to that seen in the in vitro setting in the absence of any cells.

For all scaffold formulations studied here, degradation was limited to the defect edges and scaffold exterior surface at both 12 and 18 weeks. Degradation in these
locations may be attributed to some extent to mechanical motion at the defect borders. This motion may elicit “mechanical” degradation of the non-stabilized polymer scaffold, resulting in fragmentation of the polymer network [24]. Mechanical forces external to the implant may also explain why the patterns of degradation where inconsistent even within a single scaffold. Evidently, the tissue response (bone formation) or presence of TP508 did also not influence the degradation profile of the porous PPF.

Tissue response also varied with location within a given scaffold. Overall, histological analysis showed evidence of minimal prolonged inflammatory response within the polymer scaffolds. Isolated areas of fragmentation and degradation of the polymer scaffold, however, did have elevated areas of inflammatory response cells. Degradation of PPF results in release of insoluble particulate and soluble acidic products that most likely resulted in the recruitment of the inflammatory response. It appears that the inflammatory response was not due to the presence of the scaffold, but rather the release of the degradation products. Consequently, prolonged experiments that focus on the events occurring in response to the final breakdown of the polymer scaffold are necessary for determination of the eventual success or failure of these implants.

5. Conclusions

PPF-based polymer networks exhibit many of the requirements necessary for a successful scaffold for tissue engineering. This current study examined the effects of varied release kinetics and dosages of TP508 on in vivo degradation of PPF/PLGA polymer composites. Results presented here demonstrate that minimal degradation occurred after 18 weeks in a 15.0 mm defect in the rabbit radius for all formulations tested. The extent of degradation in vivo was comparable to that observed in vitro.
### Table 7-1
Properties of PPF/PLGA Composite Scaffold Classes

<table>
<thead>
<tr>
<th></th>
<th>LB200</th>
<th>MB200</th>
<th>MB100</th>
<th>NR</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP508 dosage (µg)</td>
<td>200</td>
<td>200</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Location of</td>
<td>Pores</td>
<td>Polymer phase</td>
<td>Polymer phase</td>
<td>Polymer phase</td>
</tr>
<tr>
<td>Microparticles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-leaching</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Pluronic F127®</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

### Table 7-2
Histological Grading Scale

**Polymer degradation**
- Decrease in polymer area: 4
- Excessive fragmentation (throughout polymer): 3
- Minimal fragmentation (edges/outer-surface): 2
- Intact polymer: 1

**Tissue response within the pores of the polymer scaffold**
- Tissue in pores is mostly bone: 5
- Tissue in pores consists of bone with fibrous tissue and/or inflammatory response elements: 4
- Tissue in pores of polymer is mostly fibrous tissue, showing blood vessels and young fibroblasts invading the space with few macrophages present: 3
- Tissue in pores consists of giant cells and other inflammatory cells in abundance but connective tissue components in between: 2
- Tissue in pores is dense and exclusively of inflammatory type: 1
Figure 7-1: Histological sections of PPF/PLGA scaffolds. (a,b) Degradation was characterized by micro-fragmentation of the cross-linked network and was associated with an increase in inflammatory response; (c) multinucleated giant cells in the pores of the polymer scaffold; (d) tissue ingrowth was evident in circular pores previously occupied by PLGA microparticles.
Figure 7-2: Histological sections of PPF/PLGA scaffolds. (a,b,c) Bone ingrowth into pores of PPF/PLGA scaffold; (d) osteoblastic front inside a pore of the scaffold; (e) cartilage formation.
Figure 7-3: Results of histological scoring for polymer degradation (a) and tissue response within the pores of the implant (b) at 12 and 18 weeks. Results are means ± standard deviation for n=5.
CHAPTER VIII

Bone Regeneration in Segmental Defects in the Radius of Rabbits

1. Introduction

Bone defects arise due to a number of circumstances including trauma, disease, and congenital abnormalities. When these defects do not heal through normal biological wound healing, surgical intervention is required. The current gold standard in surgical repair uses tissue from the patient’s own body to replace the missing bone (autograft). This treatment, however, creates and secondary defect site that is open to infection. Tissue from other humans and cadavers (allografts) can also be used for transplantation, but there is often a limited supply and the potential danger of pathogen transfer exists.

Researchers in the field of tissue engineering hope to overcome the limitations of the current techniques through the development of constructs that assist the body in regenerating natural tissue even at times and in locations it would not normally do so. Techniques that are currently being investigated include the application of highly porous, biodegradable scaffolds that include the controlled delivery of growth factors and cytokines, and/or the delivery of osteogenic cell populations for complete restoration of functional tissue.

Work in our laboratory focuses on the use of poly(propylene fumarate) (PPF) based scaffolds for the controlled release of osteogenic factors for the regeneration of bone. PPF is a synthetic, linear polyester that has been extensively explored for bone tissue engineering applications. With its many unsaturated double bonds, it can be crosslinked through the use of a thermal system or UV initiation system [1, 2]. PPF breaks down due to hydrolytic degradation of its ester linkages to degradation products.
considered non-toxic [2]. PPF has been shown to support bone growth in both \textit{in vitro} and \textit{in vivo} [3-5].

To create an PPF-based osteoinductive scaffold, poly(DL-lactic-co-glycolic acid) (PLGA) microparticles carrying a bioactive protein or peptide can be added to the scaffold either through injection into the pores of a preformed PPF scaffold or though addition to the polymer phase of the scaffold prior to curing of the PPF. The release kinetics of the osteogenic peptide TP508 (Chrysalin®, MW = 22,311; AGYKDEGKRGDACEGDSGGPFV) have been shown to be controllable through altering the location of the microparticles within the scaffold as well as by other formulation parameters such as microparticle to PPF ratio and initial leachable porogen content [6].

The hypothesis of the current study is that a biodegradable polymer scaffold that serves as a carrier for an osteogenic molecule will guide bone formation and regeneration in an acute segmental defect. To test this hypothesis, four material groups were used: (1) PPF/PLGA scaffolds using a PLGA microparticles loaded with loaded with 200 μg TP508 in a Pluronic gel (PPF-200), (2) PPF/PLGA scaffolds with unloaded PLGA microparticles in a Pluronic gel (PPF-NR) (control for formulation PPF-200), (3) PLGA microparticles loaded with 200 μg TP508 in a Pluronic gel (GEL-200) (control for formulations PPF-200 and PPF-NR), and (4) unloaded PLGA microparticles and in a Pluronic gel (GEL-NR) (control for formulations PPF-200, PPF-NR, and GEL-200). Samples were retrieved 12 weeks post-implantation and bone formation was assessed using radiography, micro-computed tomography (μCT), and histomorphometry.
2. Methods and Materials

2.1 Polymer Synthesis

Synthesis of PPF and the crosslinking molecule poly(propylene fumarate) (PPF-DA) was performed as previously reported [7].

2.2 Microparticle Fabrication

Microparticles of 50:50 PLGA and polyethylene glycol (PEG) were fabricated using an established double-emulsion, solvent extraction technique ((water-in-oil)-in water) as described in [7].

2.4 PPF Scaffold Fabrication

Two formulations of PPF composite scaffolds were fabricated for this study (Table 8-1). Scaffolds were thermally crosslinked using the free radical initiator benzoyl peroxide (BP; Aldrich Chemical). An initiator solution was prepared by dissolving 1 g BP in 10 mL diethyl fumarate. PPF and PPF-DA was mixed with PPF-DA (the double bond ratio of PPF:PPF-DA was 1:2) in the presence of CH₂Cl₂ and stirred overnight at room temperature to ensure proper mixing of the two viscous solutions. After removal of the solvent from the polymer, the initiator solution (0.05 mL/g polymer) was added to the mixture with rapid stirring. The water-soluble porogen NaCl was added then to the polymer-microparticle mixture (4 g NaCl/g polymer). Finally, the resulting paste was placed on a Teflon plate and 1.6 μl N,N-dimethyl-p-toluidine (DMT, Aldrich Chemical) per gram of polymer was kneaded into the mixture. The material was inserted into Teflon molds (diameter = 4.3 mm) and placed in a 37°C oven for 20 min. The crosslinked samples were removed from the molds and placed in ddH₂O for 3 days to remove the NaCl porogen. Samples were placed on a lyophilizer to dry for 24 hours and cut to a
length of 15.0 mm. All PPF composite materials were sterilized through exposure to UV light for 12 hours prior to scaffold fabrication. All subsequent material handling occurred under sterile conditions. The complete scaffolds were exposed to UV light for an additional 12 hours after fabrication.

2.5 Pluronic Gel Preparation

Pluronic gel suspension containing microparticles was prepared as previously described [7].

2.6 Surgical Procedure

Fifteen healthy, female New Zealand white rabbits weighing between 2.1 and 2.9 kg were used in this study. Surgery was performed under general inhalation anesthesia. The anesthesia was induced by an intravenous injection of Hypnorm® (0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone) and atropine and maintained by a mixture of nitrous oxide, isoflurane, and oxygen through a constant volume ventilator. Antibiotic prophylaxis (Baytril 2.5%, Enrofloxacin, 5-10 mg/kg) was given to reduce the risk of peri-operative infection.

Animals were placed in a ventral position and immobilized on their abdomen for surgery. Hair from both legs of each animal was shaved and the skin was disinfected with povidone-iodine. A 4.5 cm longitudinal incision was made in the skin with a blade along the radius. Dissection of the muscle exposed the radius. The periosteum was removed and a 15.0 mm defect was created in the middle of the radius by dissecting the bone with a burr provided with a diamant blade under continuous saline cooling. Subsequently, the loose bone segment as well as the intervening membrana interossea was removed. Finally, the constructs were inserted in the defect site without external fixation and the muscle layers and skin were closed using a 4-0 Vicryl sutures.
For implantation of PPF-based constructs, 75 μL Pluronic/microparticle solution was injected into the pores of the PPF scaffolds and allowed to gel just prior to implantation. For Pluronic/microparticle constructs, the solution was injected directly into the defect site and allowed to gel before closure of the muscle layer.

Five constructs of each formulation were used in this study. Animals receiving implants without TP508 (NR-PPF and NR-gel) received one construct in each forelimb. Location of NR-PPF and one NR-gel was alternated with each rabbit to eliminate limb bias. Animals receiving TP508 loaded samples received just one construct in either the right or left forelimb to eliminate potential cross over effects between the two limbs. Each formulation was implanted in an equal number of left and right limbs.

At 12 weeks post-implantation, animals were euthanized with an overdose of Nembutal (pentobarbital). All constructs and surrounding tissue were retrieved en block. Dutch and United States National Institutes of Health Guidelines for the Care and Use of Laboratory Animals were observed throughout the course of this study.

2.7 Radiographic Analysis

Under light anesthesia, each rabbit was positioned prone with the forelimbs externally rotated. Serial radiographs of both forelimbs were made at 0, 8 and 12 weeks postoperatively on a Siemens Mobilett X-ray Machine.

Radiographs obtained at 12 weeks were evaluated for defect bridging and bone formation by two clinicians who were blinded to the implant type using a modified scoring system (Figure 8-1) [8]. Radiographs from week 0 were used as negative controls to assist in the location of the original defect site in the radiographs obtained at the later time points.
2.8 Micro-Computed Tomography

At the time of retrieval, bone samples and surrounding tissues were fixed in a 10% formalin solution. After fixation, all specimens were placed in PBS until further analysis could begin. To perform analysis via the μCT, samples were removed from the PBS, inserted vertically into a sample holder, and then placed in the μCT sample chamber (SkyScan 1072, SkyScan, Aartselaar, Belgium). Specimens were scanned at an energy of 80 kV, intensity of 100 μA, with a nominal resolution of 20 μm. Three samples from each formulation were chosen for analysis using μCT.

After scanning, three-dimensional images of the sections were created. As the defect edges were sometimes difficult to discern, a 5 mm wide region of interest (ROI) was isolated in the center of the defect to ensure the location of the ROI completely within the defect borders. Quantification of the bone volume per unit length was done by isolating the regenerated radius from the ulna and directly measuring the bone volume using a software program.

2.9 Histology and Histomorphometry

All samples including those analyzed with the μCT were dehydrated in a graded series of ethanol (from 70% (v/v) to 100%), and embedded in methylmethacrylate. After retrieval, specimens were prepared for histological evaluation. Specimens were fixed in 4% formalin solution (pH = 7.4), dehydrated in a graded series of ethanol (from 70% (v/v) to 100%), and embedded in methylmethacrylate. After polymerization, a minimum of three 10 μm thick sections were made longitudinally using a modified microtome technique [9]. Sections were stained with methylene blue/basic fuchsin and examined using light microscopy (Leica BV, Rijswijk, The Netherlands).
PPF-based histological sections were quantitatively assessed using a modified histological scoring analysis (Table 8-3) [7]. Samples were evaluated for: (1) bone growth into the PPF-composite scaffolds from the existing bone into the implant at the defect borders (bridging), (2) bone growth guided around the outside of the implant, (3) tissue response within the pores of the scaffold, and (4) degradation of the polymer scaffold. Two blinded reviewers evaluated at least three sections for each scaffold. The reviewers reached a consensus on the score of each section and then assigned a final score to each scaffold. The scores of the scaffolds of each formulation were then averaged to determine the overall score for the formulation. Histomorphometric analysis of bone ingrowth into the PPF-based specimens was performed to determine the maximal bone penetration distance into the PPF scaffolds. Again, at least three histological sections were digitized at low magnification and, using a Leica Qwin Pro image analysis system, the edges of the defect border were determined and the distance of bone growth into the defect was measured. The average of each scaffold was determined and then used to determine the formulation average for n= 5 scaffolds. This evaluation was only done on the PPF-based specimens because assessed parameters were not present in gel-based scaffolds.

Histomorphometric analysis was also used to evaluate the quantity of new bone formed within all of the implant types. Again, three histological sections per implant were digitized at low magnification and the defect borders were located using a Leica Qwin Pro image analysis system. Using the user defined defect edges, the computer isolated the center of the defect and sectioned out the middle 5.0 mm to define our region of interest (ROI). Within the ROI, the bone area coverage of the ulna and regenerated radius ($A_{UR}$) and the bone area coverage of the ulna ($A_U$) were measured allowing for the calculation of the bone area coverage of the regenerated radius, $A_R$, where $A_R = A_{UR} -$


A₀. The average of each scaffold was determined and then used to determine the formulation average for n= 5 scaffolds.

Statistical Analysis

A one-way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparisons test was used to determine statistical significance (p<0.05) using GraphPad InStat version 3.05 statistical software (San Diego, California, USA). Results are reported as means±standard deviation for n=3 for μCT and n=5 for histology and histomorphometry.

3. Results

3.1 X-Ray Analysis

Radiography is a noninvasive means to assess bone formation within defect sites. Radiographs were taken just after surgery and at 8 and 12 weeks. Blinded radiographic evaluation (Figure 8-2) of bone area coverage and bone union was substantial (all scores approximately 3) for both parameters examined. No significant differences were obtained, however, between samples for a given time point or between time points for a given implant formulation with this analytical technique.

3.2 Micro-Computed Tomography

Three samples from each implant group were analyzed via μCT. Using radiographs and three-dimensional reconstructions of the samples, the defect borders were approximated and the 5 mm region of interest in the center of the defect area was isolated. Three-dimensional reconstructions for the ulna and radius within the ROI for each formulation can be seen in Figure 8-3. All implant formulations are characterized
by regenerated bone spanning the ROI. Bone formation in PPF-200 and PPF-NR (Figures 8-3a and 8-3b) was characterized by growth along the outside of the scaffold. This bone appears to have a similar shape and structure to the natural radius with dense bony exterior and the formation of a central canal. Additionally, small spicules of bone can be observed within the cylindrical scaffold space, which is most likely bone ingrowth in the pores of the scaffold. Bone growth within GEL-200 and GEL-NR (Figures 8-3c and 8-3d) was located closer to the original location of the radius than in the PPF-200 and PPF-NR samples. These samples also displayed formation of a central canal within the regenerated bone.

Direct measurement of the area of bone within the radius within the ROI was also performed. Results of this analysis are shown in Figure 8-4. Although examination of the reconstructions show that the location of the bone growth varied between PPF samples and non-PPF samples, no differences in the amount of bone formed was measured.

3.3 Histology

3.3.1 Descriptive Light Microscopy Evaluation

3.3.1.1 PPF-based Implants

Histological sections are useful for the visualization of all materials within a defect site at a cellular level. Using correct staining techniques, bone, fibrous tissue, vasculature, and polymer can be easily recognized.

Figure 8-5 contains images of histological sections from both PPF-containing sample formulations (PPF-200 and PPF-NR). The polymer is easily identifiable, as it appears white even after staining of the section with methylene blue/basic fuchsine. The
structure of the pores within the scaffolds is mostly square, indicating minimal change in the scaffold macroscopic structure after implantation. Growth guided around the PPF scaffold as well as ingrowth into the scaffolds is evident in most samples, regardless of the presence of TP508 (Figure 8-5a). Active bone formation is observed within the pores of the scaffold, evidenced by osteoblast cell fronts, osteoclastic activity, and osteocytes (Figure 8-5b). In areas where bone is not present, fibrous tissue with minimum inflammatory cells is evident (Figure 8-5c). Vasculature is observable in areas of bone and fibrous tissue formation (Figures 8-5b and 8-5d).

Degradation of the polymer scaffolds is limited to the defect edges of the implants and is characterized by a localized increase in the inflammatory response (Figure 8-5e).

3.3.3.2 Pluronic/PLGA Microparticle Solution Implants

Analysis of the Pluronic/microparticle solution implants showed that neither the Pluronic nor the PLGA Microparticles were present within the defect site 12 weeks post-operatively. The original defect borders were usually easy to detect, with less mature bone appearing within the defect site (Figure 8-5f). Bone formation within the defects originated from both the ulna and defect edges. A number of samples displayed a u-shaped regenerated bone complex, with ingrowth of fibrous and muscle tissue along the external edge of the implant (Figure 8-5f). No evidence of an inflammatory response within the defect area was observed.

3.3.2 Histomorphometric Analysis

Figure 8-6 contains the quantitative results from histological scoring of PPF-based implants. Bridging of the defect border was observed in all implants examined. Histological sections showed limited bone growth around the outside of the implant. Tissue response within the pores was favorable, with a very limited inflammatory
response. Finally, PPF degradation was shown to be quite restricted in all samples. No influence of the release of TP508 from the PLGA microparticles was observed in the histological scoring analysis.

Quantification of the depth of penetration of bone in scaffolds that exhibited bridging of the defect borders can be seen in Figure 8-7. Again, no statistical difference was seen between TP508 loaded and non-loaded scaffolds implants.

Histomorphometry was also used to calculate the area of bone within a 5 mm wide region of interest located at the center of the defect area for both the two PPF-based and the two Pluronic/microparticle based formulations (Figure 8-8). No difference in the quantity of bone formed in response to the different implants was detected.

4. Discussion

The hypothesis of the current study is that a biodegradable polymer scaffold that serves as a carrier for an osteogenic molecule with guide bone formation and regeneration in an acute segmental defect. To test this hypothesis, four material groups were used: (1) PPF/PLGA scaffolds with PLGA microparticles loaded with 200 μg TP508 and a Pluronic gel (PPF-200), (2) PPF/PLGA scaffolds with PLGA unloaded PLGA microparticles and a Pluronic gel (PPF-NR) (control for formulation PPF-200), (3) PLGA microparticles loaded with 200 μg TP508 in a Pluronic gel (GEL-200) (control for formulations PPF-200 and PPF-NR), and (4) unloaded PLGA microparticles and in a Pluronic gel (GEL-NR) (control for formulations PPF-200, PPF-NR, and GEL-200). Samples were retrieved 12 weeks post-implantation and assessed using radiography, μCT, and histomorphometry.

In order to gain information regarding the quality and quantity of bone formed in the defects, a number of different analytical techniques were employed. Radiographs
were used to visualize the defect areas prior to euthanizing of the animals and harvesting the implants. Due to the two-dimensional nature of radiographs, localization and quantification of bone formed in and around a defect site can be difficult. Therefore, three-dimensional reconstructions were created using μCT. With a resolution of 20 μm, these three-dimensional reconstructions allow for imaging of the regenerated bone without inclusion of the surrounding soft tissue and implant material as well as direct measurement of bone volume. For finer resolution and visualization of all tissue and materials within the defect site, histology was performed. As μCT is non-destructive all samples used μCT analysis were thereafter used for histological analysis. Samples were embedded in polymer, cut into 10 micron sections, and viewed using light microscopy. Computer analysis software was used to quantitate average bone area within the sections of each sample.

All four formulations examined in this study showed radiographic evidence of bone formation in the defect area. μCT confirmed the presence of bone and yielded information regarding the location and supra-cellular structure of the bone. Bone formation in PPF-based formulations (PPF-200 and NR-PPF) displayed formation of bone primarily across the defect outside the area of the construct. Bone growth into the PPF porosity appeared as spicules of bone in the μCT and was confirmed using histological evaluation of the scaffold and the tissue within its pores. Apparently, bone growth into the scaffold porosity resulted in stabilization of the bone segements, creating a splinted-like situation within the defect area. Subsequently, the PPF scaffold acted as an osteoconductive surface, guiding bone across the created defect.

μCT and histology showed that the location of bone formation in response to the Pluronic/PLGA microparticle gels was different than that observed with the PPF scaffolds. In the GEL-200 and GEL-NR samples, bone formation took place not as
around the scaffold, but as ingrowth from the defect edges and the ulna. In this situation, bone formation was not due to stabilization of the implanted construct. Without a mechanically sound structure in the defect site, the defect edges were free to move. During movements, the bone segments may have been compressed to form a defect smaller than 15.0 mm in length. Degradation observed at the ends of the implanted PPF scaffold suggests compressive forces acting on the defect site during healing. This mechanical shortening of the defect appears to have been adequate to assist in closure of the defect in collaboration with the presence of the Pluronic/PLGA gel, resulting in closure of both the GEL-200 and GEL-NR samples.
Table 8-1: Implant Formulations Employed

<table>
<thead>
<tr>
<th>Scaffold Formulation</th>
<th>PPF Scaffold</th>
<th>Pluronic/ microparticle Suspension</th>
<th>TP508</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB200S</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>NRS</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>LB200G</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>NRG</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 8-2
Histological Grading Scale

Bridging of defect border\(^1\)

Yes

No

Guided bone growth (guided-growth) \(^1,2\)

76 - 100%

51 - 75%

26 - 50%

1 - 25%

= 0%

Tissue response within the pores of the polymer scaffold

Tissue in pores is mostly bone

Tissue in pores consists of bone with fibrous tissue and/or inflammatory response elements

Tissue in pores of polymer is mostly fibrous tissue, showing blood vessels and young fibroblasts invading the space with few macrophages present

Tissue in pores consists of giant cells and other inflammatory cells in abundance but connective tissue components in between

Tissue in pores is dense and exclusively of inflammatory type

Polymer degradation

Decrease in polymer area

Excessive fragmentation (throughout polymer)

Minimal fragmentation (edges/outer-surface)

Intact polymer

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Bridging of the defect border and bone growth around the scaffold was assessed with longitudinal sections only.

\(^2\) Guided bone growth (guided-growth) is a measure of the distance the bone growth around the scaffold extends from the defect border towards the center of the defect.
Figure 8-1: Schematics used to guide investigators in the quantitative evaluation of the radiographs.  (a) Radiographic evaluation of the degree of union.  Percentage values refer to the percent of the given defect border that is filled with bone that spans the defect.  (b) Radiographic evaluation of the bone formation in the defect.  Percentage values refer to the percent of the defect area that is filled with bone.
Figure 8-2: Results from radiographic scoring for the amount of bone formation within the defect area (a) and the amount of bridging from one defect border to the other. Results represent means±standard deviation for n=5.
Figure 8-3: Three-dimensional reconstructions of the 5 mm region of interest in the center of the defect. (a) PPF-200, (b) PPF-NR, (c) GEL-200 and, (d) GEL-NR.
Figure 8-4: Volume of bone per unit length (mm³/mm) for the region of interest as determined by μCT. Results are presented as means±standard deviations for n=3.
Figure 8-5: Histological sections. (a, b) Bone growth around the PPF-based implant; (b) evidence of bone formation (osteoblasts, osteoclasts, and osteocytes) in the pores of the PPF-based scaffold; (c) fibrous tissue with minimal inflammation in the pores of the PPF-based scaffold; (a, d) vasculature; (e) inflammatory cells in association with PPF-based polymer fragmentation; (f) typical structure of bone formation in defects loaded with a Pluronic/microparticle suspension.
Figure 8-6: Quantitative results of histological analysis using the scoring system presented in Table 8-3. (a) Bridging of the defect border, (b) bone growth guided around PPF scaffold, (c) tissue response in the pores of the scaffold and, (d) polymer degradation.
Figure 8-7: Average maximum distance of bone penetration into the polymer scaffold for the two polymer scaffold formulations. Results are means ± standard deviation for n=5.
Figure 8-8: Area of bone per unit length (mm²/mm) for the region of interest as determined by μCT. Results are presented as means ± standard deviations for n=3.
CHAPTER IX

Controlled Release of Hyaluronan Oligomers from Biodegradable Polymeric Microparticle Carriers*

Abstract

Hyaluronan (HY) oligomers of varying molecular weights were incorporated into biodegradable microparticles of blends of poly(DL-lactic-co-glycolic acid) (PLGA) and poly(ethylene glycol) (PEG). Using a 2-level fractional factorial experimental design, four microparticle formulation parameters, the amount of PEG included in the microparticles, the initial HY loading of the microparticles, the molecular weight of HY, and the molecular weight of PLGA, were studied for their influence on the release kinetics of HY in vitro over the period of 28 days. The entrapment efficiencies were found to range between 10.4 ± 1.3% and 16.5 ± 0.8% depending on the initial loading and the molecular weight of the HY oligomer used in the fabrication of the microparticles. The HY was released in a multiphasic fashion including an initial burst release, followed by two separate periods of linear release. The normalized cumulative mass released during the burst release ranged from 25.1 ± 9.4% to 92.9 ± 1.0% and was found to be significantly influenced by the initial HY loading, the HY molecular weight, and the PLGA molecular weight. The initial period of linear release lasted from day 1 to day 14 and displayed normalized cumulative rates of release from 0.001 ± 0.000 day\(^{-1}\) to 0.014 ± 0.002 day\(^{-1}\). During this period, PEG content of the microparticles and HY molecular weight exerted the greatest influence on the rate of release. Finally, the second period of linear release lasted through the final time-point at day 28. Here, the normalized cumulative rate of release values ranged from 0.002 ± 0.000 day\(^{-1}\) to 0.036 ± 0.007 day\(^{-1}\) and were

* This chapter has been submitted for publication as: E.L. Hedberg, C.K. Shih, L.A. Solchaga, A.I. Caplan, A.G. Mikos, "Controlled release of hyaluronan oligomers from biodegradable polymeric microparticle carriers," J Control Rel.
dependent on all formulation parameters studied. These results demonstrate the potential of PLGA/PEG blend microparticles for the controlled release of HY oligomers.

1. Introduction

There has been interest in the use of microparticle carriers for the localized, controlled delivery of bioactive factors into orthopedic defect sites. One of the commonly employed systems is microparticles fabricated from poly(lactic-co-glycolic acid) (PLGA). These copolymers are attractive for this application due to their many favorable characteristics including good biocompatibility and the ability to degrade into natural metabolites [1-3]. PLGA microparticles have been used for the localized delivery of many bioactive molecules including osteoinductive growth factors such as transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein-2 (BMP-2) [4-8]. Altering the microparticle composition allows for modulation of the release kinetics of the incorporated proteins. The molecular weight of the PLGA, the ratio of lactic to glycolic acid units within the PLGA, and the addition of the more hydrophilic polymer poly(ethylene glycol) (PEG) to create PLGA/PEG blend microparticles are factors that have been shown to influence release of bioactive factors from PLGA-based microparticles [2, 8, 9].

Hyaluronan (HY) is a naturally occurring polysaccharide that consists of repeating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid. It constitutes a major component of the extracellular matrix, particularly within connective tissues, and is found abundantly in the vitreous and synovial fluid [10]. As HY is biocompatible and biodegradable, it has been used as a scaffold material for tissue engineering [11].

Recent work has shown that the use of two HY-based scaffolds, ACP and HYAFF-11 sponges, in full thickness defects in the rabbit knee aided in the regeneration
of both the bone and cartilage layers [12, 13]. Chemical differences in these two HY-based scaffolds resulted in differing in pore sizes and degradation periods. ACP sponges had an average pore diameter of approximately 26 μm and an in vivo half-life of approximately 1 week; HYAFF-11 sponges had an average pore diameter of approximately 83 μm and an in vivo half-life of approximately 4-5 weeks. These studies showed that the ACP sponges had a slightly higher success rate in regenerating tissue than the HYAFF-11 sponges [12].

In order determine the mechanism driving the success of the HY-based scaffolds, further studies were subsequently performed to assess the effects of the scaffold morphology and rate of degradation on tissue formation in the full-thickness defect in the rabbit knee. PLGA scaffolds with similar pore morphologies and rates of degradation to the ACP scaffolds were fabricated along with poly(L-lactic acid) (PLLA) scaffolds that had similar pore morphologies and rates of degradation to the HYAFF-11 scaffolds. These polymer scaffolds were compared to the corresponding HY-based scaffolds in terms of successful repair of full thickness defects to determine the effects of pore morphology and rate of degradation of these scaffolds that aided in tissue regeneration. Results showed both HY scaffolds exhibited greater effectiveness when compared to the PLGA and PLLA scaffolds [13].

HY has been found in a range of naturally occurring molecular sizes, from several hundred to over 10 million Daltons (MDa). The average size of HY is approximately 1-2 MDa [14]. Smaller fragments of HY (oligosaccharides) exhibit different specific cell-biological properties as compared to the larger, mega Dalton-sized biopolymer. For example, HY oligomers have displayed angiogenic potential [14]. In order to investigate the effect of the HY scaffold degradation products on the treatment of full thickness defects in articular cartilage, a proposed design combines PLGA and
PLLA scaffolds with pore morphologies and rates of degradation similar to HY scaffolds with a PLGA microparticle drug release system of HY oligomers. The ability to control the release kinetics of HY from a PLGA microparticle carrier will allow us to investigate the effects of the varied release profiles of HY on tissue formation.

The aim of this study was to determine what fabrication parameters of PLGA microparticles influenced the release of HY oligomers. Using a fractional factorial design, the amount of the more water soluble polymer poly(ethylene glycol) (PEG) included into the microparticles, the initial HY loading of the microparticles, the molecular weight of HY, and the molecular weight of PLGA were studied for their influence on the release of HY over the period of 28 days. The main effects of each parameter on the HY release kinetics were then determined.

2. Materials

Two molecular weights of PLGA with lactic to glycolic acid copolymer ratios of 50:50 were used in this study (Medisorb®, Alkermes, Cincinnati, OH). Gel permeation chromatography was employed to determine the weight average molecular weight ($M_w$) and the polydispersity index of each PLGA sample, where the PI is equal to the ratio of the weight average molecular weight to the number average molecular weight ($M_w/M_n$). The low level molecular weight PLGA had a $M_w$ of 47,600 kDa and a PI of 1.73, while the high level molecular weight had a $M_w$ of 75,500 kDa and a PI of 2.03. Poly(ethylene glycol) (PEG, $M_w$=10,700) and poly(vinyl alcohol) (PVA 88 mol% hydrolyzed, $M_w$=13,000-23,000) were purchased from Aldrich Chemical (Milwaukee, WI). Sodium hylauronate oligomers of two molecular weights ($M_w$=15,000, PI=1.23 and $M_w$=49,500, PI= 1.18) were received from Lifecore Biomedical (Chaska, MN) as lyophilized powders. Phosphate buffered saline (PBS) was obtained from Gibco (Grand Island, NY). Sodium
tetraborahydrate, sulfuric acid, and carbazole were purchased from Aldrich Chemical and used as received.

3. Methods

3.1 Experimental Design

HY release kinetics were studied using a fractional factorial design [15, 16] varying 4 factors: PEG content of the microparticles (MP), initial HY loading, HY molecular weight, and PLGA molecular weight. The high and low values for each parameter are presented in Table 9-1(a). The formulation combinations are presented in Table 9-1(b). These eight formulations of PLGA microparticles were placed in 1.8 ml eppendorf tubes and suspended in 1.5 ml of PBS. Vials were incubated on an orbital shaker at 37° C. At 12 hr, 1, 4, 7, 14, 17, 21, and 28 days samples were centrifuged (180 x g for 1 min) and supernatant was removed. Microparticles were resuspended in fresh PBS and returned to 37° C with agitation.

Eight formulations corresponding to the parameters listed in Table 9-1(b) but without HY within the microparticles constituted the control PLGA microparticles that were run in parallel with the HY-loaded microparticles for use in the modified HY carbazole assay.

3.2 Microparticle Fabrication and Characterization

PLGA microparticles were fabricated using an established ((water-oil)-water) emulsion technique [17]. Briefly, HY oligomers were dissolved in 125 µl of water and vortexed with PLGA and PEG dissolved in methylene chloride for 1 min. Then, 1.5 ml of a 0.3% PVA solution was added to the emulsion and vortexed for another 1 min. This
second emulsion was poured into a 250 ml beaker with 99.5 ml of PVA and 100 ml isopropyl alcohol (2% in ddH$_2$O) and stirred with a 50 mm stir bar for 1 hour at 270 rpm. The formed microparticles were harvested and dried using centrifugation at 180xg for 1 min and lyophilized for 12 hrs.

Prior to initiation of the release study, the average diameter for each microparticle formulation was measured using a Multisizer 3 Coulter Counter (Beckman Coulter, Fullerton, CA). To calculate the entrapment efficiency of HY within the microparticles, the theoretical and actual loads were determined. The theoretical load was calculated by dividing the initial mass of HY added to the microparticle fabrication by the mass of microparticles fabricated per batch. The actual load was determined by dissolving 20 mg of the loaded microparticles in 1 ml of methylene chloride at 37°C with agitation [8]. After 6 hours, 1 ml of PBS solution was added to extract the water-soluble HY. A modified Bitter and Muir carbazole assay and corresponding HY standard curve was used to determine the mass of HY entrapped ($M_e$) [18, 19].

3.3 HY Standard Solutions

In fabricating the HY standard solutions, PLGA degradation products for each corresponding time point were introduced into the standards used to determine the concentration of the samples collected at those time points. This was done to account for any effect that the degradation products of the microparticles could have on the assays. HY standards including blank PLGA microparticle supernatant were constructed by dissolving HY (8 mg) in ddH$_2$O (8 ml) to form a stock solution. The HY stock solution was then diluted with a combination of water and 0.5 ml of degradation products to make 1 ml standards with the final concentrations [0, 0.04, 0.08, 0.12, 0.16, 0.20, 0.24, 0.28 mg HY/ml] used for the standard curve.
3.4 Hyaluronan Carbazole Assay

An established modified Bitter and Muir carbazole assay was performed [18, 19]. Sodium tetraborohydrate was made by dissolving 191 mg of sodium tetraborohydrate in 20 ml of sulfuric acid. A 0.125% (w/w) carbazole solution was made by dissolving 0.0125 g of carbazole in 9.9875 g of ethanol. Sodium tetraborohydrate (3 ml) was placed in test tubes and cooled to 4°C in a constant temperature chamber. While in the chamber, the HY solutions (0.5 ml) were carefully layered over the sodium tetraborohydrate and the closed tubes were first shaken gently and then vigorously. Tubes were subsequently heated for 10 min in a boiling hot water bath followed by cooling to room temperature. Carbazole (0.1 ml) was then added to the tubes with shaking. The samples were heated again in a boiling water bath for 15 min followed by cooling to room temperature. Three hundred microliters (300 μL) of the resulting solution were added to 96 well plates and the absorption of the samples were measured at 530 nm on a plate reader (FLx800; BIO-TEK Instruments, Winooski, VT). A standard curve was used to obtain the concentrations of sample HY solutions. Standard solutions over a known concentration range were run and the obtained absorbance was plotted against the known concentration to create a standard curve. The concentration for the sample was then calculated using the standard curve. The total amount of HY released for any given time point was then calculated by multiplying the sample concentration by the sample volume.

To determine the cumulative mass released for each time-point, the HY cumulative mass released through any given time-point \( (M_t) \) was obtained by adding the total amount of HY released for each prior time-point to that time-point. The normalized cumulative mass was then obtained by dividing \( M_t \) by \( M_{50} \). Finally, the rate of release
was considered to be the slope of the normalized cumulative mass release curve. The slope of the curve for each distinct region of release was determined using linear regression.

Statistical Analysis

In analyzing the effects, the standard error of the mean was determined for each measured response [15, 16]. An effect was considered significant if the mean value of the effect was larger than the standard error of the mean.

4. Results

4.1 PLGA Microparticle Characterization

No difference in microparticle diameter was observed between formulations with average diameters ranging from 16.0±2.4–17±2.1 μm. The entrapment efficiencies are given in Figure 9-1. The entrapment efficiency main effects were calculated for each parameter and are presented in 9-2. Neither the PEG content nor PLGA molecular weight had a significant effect on the entrapment efficiency of HY within the microparticles. The molecular weight of the HY as well as the percent loading of the HY in the microparticle formulations did have an effect with the HY molecular weight exerting the greatest influence.

4.2 HY Oligomer Release

In order to compare the release profiles of the varying MP formulations, the cumulative mass release of HY for each time point (Mt) was calculated and normalized by the total amount of HY loaded in within the microparticles (M∞). The release results
show variation among the different microparticle formulations (Figure 9-3). Three regions of release were observed: an initial burst release lasting until day 1, a linear region of release from day 1 through 14, and a second region of linear release from day 14 through day 28.

The calculated rate of release through day 1 (the burst release) can be seen in 9-4. The main effect of each parameter is presented in Figure 9-5 for the burst region of release. Varying HY loading, HY molecular weight, and PLGA molecular weight all influenced the rate of release.

The rates of release of HY during the first region of linear release extending from day 1 to day 14 (Figure 9-6) ranged from 0.001 to 0.014 day$^{-1}$. The calculated main effects (Figure 9-7) for this region show that PEG content, HY molecular weight, and PLGA molecular weight had a significant influence on the rate of release.

The rate of release during the second region of linear release from day 14 through the final time point at day 28 (Figure 9-8) have values ranging from 0.002 to 0.036 day$^{-1}$. The calculated main effects (Figure 9-9) demonstrate that PEG content, HY loading, HY molecular weight, and PLGA molecular weight all influence the rate of release during this final phase.

5. Discussion

PLGA microparticles have been extensively used for the controlled release of many proteins and peptides. In the study presented here, PLGA microparticles were explored for the delivery of oligomers of the polysaccharide HY. Using a $2^{4-1}$ fractional factorial designed experiment, the effect of varying poly(ethylene glycol) (PEG) content, initial HY loading, HY molecular weight, and PLGA molecular weight on the release of HY oligomers from PLGA/PEG microparticles over the course of 28 days was determined.
The entrapment efficiency of HY within the PLGA microparticles was found to be determined mainly by the initial loading and molecular weight of HY. An increase in the initial loading of HY within the PLGA microparticles reduced the entrapment efficiency of the HY. This effect has been observed in a number of previous studies with microparticles fabricated using a ((water-oil)-water) technique [20]. With this type of method, an aqueous solution of a water soluble drug is injected into a non-aqueous solution in which the polymer but not the drug is soluble. Through agitation of the mixture, small droplets of the first aqueous phase are distributed throughout the external oil phase. Upon the addition of another, external aqueous phase, the oil phase separates from the external water phase while still containing the internal water phase and drug loaded microparticles are formed. If the concentration of the target drug is high within the internal water phase, the concentration gradient between the internal and external water phases may be great enough to drive the movement of HY through the oil phase to the external water phase. This increase of drug concentration gradient between the drug emulsion phase and the outside water phase of fabrication increases the amount of drug dissolved into the outside water phase, thereby lowering the amount of drug encapsulated within the PLGA oil phase and, subsequently, the PLGA microparticles [20]. An increase in HY molecular weight, however, increased in the entrapment efficiency. Increasing HY molecular weight decreases the solubility of HY; therefore increasing the molecular weight of HY decreased the dissolution of the drug from within the drug emulsion phase into the outside water phase during fabrication.

The cumulative mass release of HY from PLGA microparticles during the initial burst was influenced by the initial loading of HY, the molecular weight of the HY, and the PLGA molecular weight. Initial release from PLGA microparticles can be attributed to the HY that is located at or near the surface of the microparticle. Contact with the surrounding environment readily dissolves the exposed HY [17]. In this study, an
increase of the HY initially loaded into the microparticles increased the burst. The increase in HY loading leads to a sharper concentration gradient of HY between the PLGA microparticles and the aqueous solution surrounding the microparticle. A higher initial load of HY also increases the potential of a larger amount of HY distributed on the surface of the microparticles. The sharper gradient promotes the movement of from inside the microparticles into the outer, aqueous phase [20]. Therefore, a larger amount of HY distributed on the surface of the microparticles increases the rate of dissolution for HY from the surface. As previously seen with proteins of various molecular weights [3], increasing the molecular weight of the HY entrapped in the particles was shown to result in a decrease of the initial burst release. Higher molecular weight HY dissolves at a slower rate than lower molecular weight HY, thereby decreasing the rate of dissolution of the HY located on the surface of the microparticles. Additionally, for molecules with linear conformations such as HY, increasing the molecular weight may enhance the entrapment of part of the oligomer in the PLGA matrix. When this occurs, the initial burst release is reduced, as the release of partially embedded HY is dependent on the diffusion of the entrapped segment of the HY out of the matrix despite the molecule’s early exposure to the aqueous environment.

A distinct region of linear release was observed from day 1 through day 14. In this region it was found that the initial PEG content, HY molecular weight, and PLGA molecular weight influenced the rate of release. The calculated main effects show that an increase in initial PEG composition within the PLGA microparticles decreases the rate of release of HY during this period. While it is thought that, due to its hydrophilicity, incorporating PEG into the microparticles should increase their degradation and thereby increase the release of HY, it has been shown that inclusion of PEG can, sometimes, decrease the rate of release during the period following the initial burst release [9]. PLGA molecular weight was also shown to influence the rate of release during the initial
plateau. By increasing the molecular weight of the PLGA, the rate of release is decreased. Higher molecular weight PLGA has longer polymer chains, leading to greater entanglement between PLGA chains. This increased entanglement slows the diffusion of HY through the PLGA matrix. Conversely, PLGA with a lower molecular weight has shorter chains and less entanglement among the chains. During the initial plateau the PLGA undergoes random chain scission through hydrolysis of its ester linkages [2]. As this cleavage occurs, entanglement of the chains decreases, and the HY is diffuses out of the microparticles more readily. Since lower molecular weight PLGA has less chain entanglement, the HY diffuses out of the microparticles more readily when compared to microparticles made of more entangled, higher molecular weight PLGA. Additionally, cleavage may be slower for high molecular weight PLGA microparticles because a more entangled network allows for slower water influx.

The third region of release, days 14 through 28, is characterized by an increase in the rate of release. In this region all factors examined in this study, PEG content, HY initial loading, HY molecular weight, and PLGA molecular weight influenced the rate of release. An increase in the PEG content here was determined to increase the rate of release of HY from the PLGA microparticles. Since the hydrophilic PEG is released from the microparticle faster than the surrounding PLGA, channels within the PLGA network are created facilitating the access from the surrounding aqueous environment, thereby helping to increase the degradation of the PLGA. When the PLGA degrades more quickly, HY diffuses out more rapidly of the microparticles.

The release kinetics of a number of different molecules from PLGA/PEG blend microparticles have been reported previously [8, 9, 17]. Taken together these studies demonstrate that the release of a given compound from a microparticle is dependent not only on the formulation of the microparticle but on the characteristics of the incorporated factor as well. For example, an increase in the initial PEG content from 0 to 5 wt% has
been shown to increase the initial cumulative mass release of FITC-dextran (M_w=19,600 kDa), [9] but decrease the release of TGF-β1 under the same conditions [8].

Comparing polysaccharides of molecular weights similar to the HY examined here (FITC-dextran, M_w=19,600 kDa; HY, M_w=15,000 kDa and M_w= 49,500 kDa), a much lower entrapment efficiency for HY is obtained than for FITC-dextran. Additionally, the release profiles were substantially different. FITC-dextran exhibited 4 regions of release over the course of 28 days characterized by a large second burst release between days 10 and 14. HY only displayed 3 regions of release, with no secondary burst release for any formulation over the same time period.

The 3-region profile was observed for the release of the 23 amino acid peptide TP508 (M_w= 2,300) from PLGA/PEG blend microparticles [17]. However, the influence of each formulation parameter on the release kinetics was not always consistent between TP508 and HY loaded microparticles. While an increase in the initial drug loading resulted in an increase in the rate of release through day 1 of both bioactive molecules, an increase in PEG led to an increase in the rate of release of TP508 but not in the rate of release of HY over the same time period. This difference in response to the addition of PEG to the microparticle formulation may be attributed to the difference in diffusivity of the molecules through the pores created by the initial loss of the water-soluble PEG from the microparticle due to the size difference between the HY oligomers and TP508.

Results of this study demonstrate that HY oligomers can successfully be incorporated into PLGA microparticles and that by altering the PEG content, PLGA molecular weight, and HY initial loading the release kinetics of the HY oligomers of varied molecular weights can be modulated. Using these results, the desired release profiles mimicking the release of degradation products from a HY based scaffolds can be
designed, allowing for the assessment of the effect of varied release kinetics of HY oligomers on tissue regeneration in full-thickness cartilage defects.

5. Conclusions

Variation in the formulation of PLGA microparticles leads to alternations in the release kinetics of incorporated HY of oligomers of different molecular weights. All formulations examined in this study exhibited three regions of release, a burst release lasting through day 1, an initial region of linear release from day 1 through day 14, and a second region of linear release from day 14 though the final day of the study, day 28. Changing the percentage of PEG within the microparticle showed no effect on the cumulative mass released during the initial burst release, but did influence the rates of release during both the first and second regions of linear release. The initial loading of the HY within the microparticles and the PLGA molecular weight, as well as the molecular weight of the HY itself, were shown to influence all regions of release.
Table 9-1: Microparticle Experimental Design

(a) High and low values for the parameters tested in the $2^{4-1}$ fractional factorial design

<table>
<thead>
<tr>
<th></th>
<th>PEG Content (mg PEG/mg MP)</th>
<th>HY Initial Loading (mg HY/mg MP)</th>
<th>HY MW</th>
<th>PLGA MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Level (+)</td>
<td>5%</td>
<td>3%</td>
<td>140,000</td>
<td>75,500</td>
</tr>
<tr>
<td>Low Level (-)</td>
<td>0%</td>
<td>1%</td>
<td>14,000</td>
<td>47,600</td>
</tr>
</tbody>
</table>

(b) Combinations of the parameters in the $2^{4-1}$ fractional factorial design

<table>
<thead>
<tr>
<th></th>
<th>PEG Content (mg PEG/mg MP)</th>
<th>HY Initial Loading (mg HY/mg MP)</th>
<th>HY MW</th>
<th>PLGA MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>M3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M4</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M5</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M6</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M7</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 9-1: Entrapment efficiency of HY oligomers within PLGA microparticles using an established ((water-oil)-water) technique. The results are presented as means ± standard deviation for n = 3.
Figure 9-2: Main effects of PEG content, HY loading, HY molecular weight, and PLGA molecular weight on HY entrapment efficiency within PLGA microparticles. A positive number indicates an increase in the entrapment efficiency with a change from the low level to high level for the individual parameters and a negative number indicates a decrease in the rate of release. The results are presented as means ± standard error of effect for n = 3.
Figure 9-3: Cumulative release of HY from PLGA/PEG microparticles in PBS at 37°C. The release is expressed as normalized mass release for all time points. The results are presented as means ± standard deviation for n = 5.
**Figure 9-4**: Rate of release of HY from the PLGA/PEG microparticles during the 1-day burst release period. The results are presented as means ± standard deviation for $n = 5$. 
**Figure 9-5**: Main effects of the PEG content, HY loading, HY molecular weight, and PLGA molecular weight on the rate of release during the first day. A positive number indicates an increase in the rate of release with a change from the low level to high level for the individual parameters and a negative number indicates a decrease in the rate of release. The results are presented as means ± standard error of effect for n = 5.
Figure 9-6: Rate of release of HY from the PLGA/PEG microparticles in PBS during the day 1 through day 14 time period. The results are presented as means ± standard deviation for n = 5.
Figure 9-7: Main effects of the PEG content, HY loading, HY molecular weight, and PLGA molecular weight on the rate of release period during the day 1 through day 14 time period. A positive number indicates an increase in the rate of release with a change from the low level to high level for the individual parameters and a negative number indicates a decrease in the rate of release. The results are presented as means ± standard error of effect for n = 5.
Figure 9-8: Rate of release of HY from the PLGA/PEG microparticles in PBS during the day 14 through day 28 time period. The results are presented as means ± standard deviation for n = 5.
Figure 9-9: Main effects of the PEG content, HA loading, HA molecular weight, and PLGA molecular weight on the rate of release during the day 14 through day 28 time period. A positive number indicates an increase in the rate of release with a change from the low level to high level for the individual parameters and a negative number indicates a decrease in the rate of release. The results are presented as means ± standard error of effect for n = 5.
CHAPTER X

rhBMP-2 Release from Injectable Poly(DL-Lactic-co-Glycolic Acid)/Calcium Phosphate Cement Composites

Abstract

**Background:** In bone tissue engineering, poly(DL-lactic-co-glycolic acid) (PLGA) microparticles are frequently used as delivery vehicle for bioactive molecules. Calcium phosphate (Ca-P) cement is an injectable, osteoconductive and degradable bone cement that sets in situ. The objective of this study was to create an injectable composite based on Ca-P cement embedded with PLGA microparticles for sustained delivery of recombinant human bone morphogenetic protein-2 (rhBMP-2).

**Methods:** $^{125}$I labeled rhBMP-2 was incorporated in PLGA microparticles. PLGA microparticles/Ca-P cement composites were prepared in a 30:70 wt% ratio. Material properties were evaluated by SEM, μCT and mechanical testing. Release kinetics of rhBMP-2 from PLGA/Ca-P cement discs and PLGA microparticles alone were determined in vitro in two buffer solutions (pH 7.4 and pH 4.0) for up to 28 days.

**Results:** The entrapment yield of rhBMP-2 in PLGA microparticles was 79 (±8)%.

Analysis showed spherical PLGA microparticles (average size of 17.2 [±1.3] μm) distributed homogeneously throughout the nanoporous discs. The compressive strength was significantly lower for PLGA/Ca-P cement composite scaffolds than for Ca-P scaffolds alone (6.4 [±0.6] MPa vs. 38.6 [±2.6] MPa, respectively). Average rhBMP-2 loading was 5.0 (±0.4) μg per 75 mm³ disc. Release of rhBMP-2 was limited for all formulations. At pH 7.4, 3.1 (±0.1)% of the rhBMP-2 was released from the PLGA/Ca-P

---


* Authors contributed equally.
cement discs and 18.0 (±1.9)% from the PLGA microparticles alone after 28 days. At pH 4.0, PLGA/Ca-P discs revealed more release of rhBMP-2 than PLGA microparticles alone (14.5 [±6.3]% vs. 5.4 [±0.7]%) by day 28.

**Conclusions:** The results in the current study indicate that preparation of PLGA/Ca-P cement composite for the delivery of rhBMP-2 is feasible and that the release of rhBMP-2 is dependent on the composite composition and nanostructure as well as the pH of the release medium.

**Clinical Relevance:** An osteoconductive and osteoinductive, rhBMP-2 loaded PLGA/Ca-P cement composite will potentially result in an injectable bone graft substitute for regeneration of bone in ectopic or orthotopic sites.

1. **Introduction**

Porous biodegradable polymers and ceramics are frequently used as scaffold materials in bone tissue engineering approaches. Among the various available biodegradable polymers, aliphatic polyesters of polylactic acid, polyglycolic acid, and poly(DL-lactic-co-glycolic acid) (PLGA) are popular because of their long clinical experience, favorable degradation characteristics and possibilities for sustained drug delivery. PLGA can be processed into spherical microparticles for delivery of bioactive molecules. In light of this, PLGA microparticles have been thoroughly investigated as delivery vehicles for transforming growth factor-β1 (TGF-β1), recombinant human bone morphogenetic protein-2 (rhBMP-2), and other molecules [1-6]. Polymer degradation and drug release rates from these microparticles can be adjusted by altering the polymer molecular weight, copolymer ratio and the microparticle size and structure. These rates are also influenced by environmental factors such as pH which can decrease because of physiological (inflammation phase of wound healing) or iatrogenic (PLGA degradation products) conditions.
Calcium phosphate (calcium-phosphate) materials are widely used as bone substitutes in orthopaedic, reconstructive, and oral surgery due to their beneficial effect on bone healing. Manufacturing of calcium-phosphate ceramics usually requires processing at elevated temperatures. Most calcium-phosphate ceramics, therefore, are available for implantation only as granules or prefabricated blocks. Unfortunately, granules can easily migrate into the surrounding tissue and prefabricated blocks are difficult to shape and may result in incomplete filling of the bone defect. A solution for these processing and delivery problems can be found in an injectable calcium-phosphate cement that can be shaped according to the defect dimensions. Although injectable calcium-phosphate cement reveals excellent biocompatibility and osteoconductivity, in vivo resorption and tissue ingrowth is slow [7]. These limitations may be overcome by the generation of a macroporous structure within a Calcium-phosphate implant [7]. Compressive strength of a macroporous cement, however, is greatly decreased [8].

Growth factors have been added to injectable calcium-phosphate ceramics either before setting of the cement [9-11] or after complete setting [12,13]. In those studies, a solution containing the growth factor was directly mixed with the calcium phosphate powder or adsorbed onto the cement surface. When in vitro release was determined, it was shown to be rather slow (3-13% after two weeks) [9,11,13]. In the case of a non-macroporous structure, prolonged retention of entrapped proteins might result in the loss of their osteoinductive potential. Therefore, we presume that delivery of growth factors from injectable calcium-phosphate cements can be optimized.

We hypothesize that incorporation of rhBMP-2 loaded microparticles in calcium-phosphate cement is feasible and will result in a sustained release of bioactive protein under various conditions. The aim of our research is to develop an injectable composite based on calcium-phosphate cement with PLGA microparticles as the delivery vehicle.
for rhBMP-2. Controlled release of the growth factor from the microparticles should result in a continuous level of bioactive protein delivered in the surrounding tissues. Also, degradation of the PLGA microparticles should result in a macroporous calcium-phosphate cement structure that allows for increased bone in-growth. Additionally, the resulting macroporosity should expedite the degradation of the calcium-phosphate cement scaffold. In the current study, we focus on the composite preparation and determination of the rhBMP-2 release kinetics.

2. Materials and methods

2.1 Materials

RhBMP-2 was supplied by Yamanouchi Europe BV., Leiderdorp, The Netherlands. $^{125}$I (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was used to label rhBMP-2 with the Iodo-Gen® tube according to the manufacturer’s instructions (Pierce, Rockford, IL, USA). Iodination was kindly done by Chrysalis Biotechnology Inc., Galveston TX, USA. Poly(DL-lactic-co-glycolic acid) (PLGA) (Medisorb; Alkermes, Cincinnati, Ohio) with a 50:50 lactic to glycolic acid copolymer ratio was used. Gel permeation chromatography was used to determine the weight average molecular weight of four samples, which was 47.6 ± 0.5 kDa, and the mean polydispersity index (the ratio of weight average and number average molecular weights [Mw/Mn]), which was 1.73 ± 0.05. Ancillary chemicals for microparticle preparation were all of analytical grade and obtained as previously described. The powder component of the calcium phosphate cement (Calcibon; Biomet Merck, Darmstadt, Germany) consisted of a mixture of 62.5% by weight tricalcium phosphate (α-TCP), 26.8% by weight dicalcium phosphate anhydrous (DCPA), 8.9% by weight calcium carbonate (CaCO$_3$) and 1.8% by weight
hydroxyapatite (Hap). An aqueous solution of Na₂HPO₄ (2% by weight) was used as the liquid component.

2.2 Preparation of ¹²⁵I rhBMP-2 Loaded PLGA Microparticles

The rhBMP-2 solution used in this study was produced by adding a trace amount of ¹²⁵I labeled rhBMP-2 to an unlabeled rhBMP-2 solution (hot:cold rhBMP-2 = 1:100). The hot/cold rhBMP-2 solution was diluted in a buffer solution of phosphate buffered saline and bovine serum albumin (PBS/BSA[0.1%]) to a final concentration of 1.6 mg/ml. PLGA microparticles were prepared by a double-emulsion-solvent-extraction technique ([water-in-oil]-in-water) as previously described [1,6,14]. The microparticles were produced by injecting 125 µl hot/cold solution (200 µg rhBMP-2) into a flint glass tube containing a solution of 242.3 mg PLGA in 1 ml dichloromethane. This mixture was emulsified for 60 sec on a vortexer (Vortex Genie 2, Scientific Industries, Bohemia, NY, USA). Subsequently, 1.5 ml 0.3% aqueous poly(vinyl alcohol) (PVA) solution was added and emulsified for 60 sec on a vortexer to produce the second emulsion. The mixture was then added to 98.5 ml PVA solution and 100 ml of 0.2% aqueous isopropanol solution with rapid stirring for 1 hr. The extraction of the dichloromethane into the external aqueous phase resulted in the precipitation of the dissolved polymer and subsequent microparticle formation. The formed microparticles were collected, centrifuged at 1000 rpm for two minutes, lyophilized, weighed, and stored at -20⁰C until use. The entrapment efficiency of the protein was determined by normalizing the amount actually entrapped with the amount added to the fabrication process. A calibration curve was generated through serial dilutions of the ¹²⁵I-rhBMP-2 stock solution to determine the quantity of rhBMP-2 (µg/mg microparticle), with the use of a γ-counter (Cobra II Autogamma, Packard, Meridian, CT, USA).
2.3 Preparation and Characterization of Blank PLGA Microparticles

Blank microparticles were prepared according to the previously described protocol, with use of PBS/BSA[0.1%] without rhBMP-2 as the first aqueous phase. The size of the microparticles was measured with a Coulter counter multisizer (model 0646, Coulter Electronics, Hialeah, FL, USA). The morphology of the microparticles was evaluated by scanning electron microscopy (SEM) (Jeol JSM-5300 Scanning Microscope; JEOL, Peabody, Massachusetts).

2.4 Preparation of $^{125}$I-rhBMP-2 PLGA/Calcium-Phosphate Cement Composite

For the preparation of $^{125}$I-rhBMP-2 loaded PLGA/calcium-phosphate cement discs, a mixture of blank PLGA microparticles and $^{125}$I-rhBMP-2 loaded PLGA microparticles (50:50 by weight) was used to achieve a rhBMP-2 dosage of approximately 5 $\mu$g per disc. This mixture of PLGA microparticles was added to the calcium-phosphate cement powder in a PLGA:calcium-phosphate weight ratio of 30:70. A 3 ml syringe (Becton Dickinson, Franklin Lakes, New Jersey) was closed at the tip with a plastic stopper and filled with 700 mg of this PLGA/calcium-phosphate mixture. After 300 $\mu$l of Na$_2$HPO$_4$ (2% by weight) was added, the syringe was closed with the injection plunger and was placed in a mixing apparatus (Silamat; Vivadent, Schaan, Lichtenstein). After mixing for fifteen seconds, the stopper was removed and the composite was injected in Teflon molds (6.35 mm in diameter and 2.4 mm in thickness). The disks were removed from the molds after setting of the cement at room temperature for one hour. Radioactivity in the disks was determined with the use of a counter to quantify the initial amount of rhBMP-2 per disk.
2.5 Preparation and Characterization of Blank PLGA/Calcium-Phosphate Cement Composite

Similar to preparation of the $^{125}$I-rhBMP-2 loaded PLGA/calcium-phosphate cement discs, blank PLGA/calcium-phosphate cement and control discs (Calcium-phosphate cement without PLGA microparticles) were prepared. The specimens were used for calcium-phosphate cement cytotoxicity assessment, analysis by SEM and microcomputed tomography ($\mu$CT) (Scanco Medical, $\mu$CT 80, resolution 10 $\mu$m, Bassersdorf, Switzerland) and mechanical testing (scaffold dimensions 6.0 mm diameter and 12.0 mm height). Discs used for cytotoxicity assessment were sterilized by autoclaving for 15 min at 121°C.

2.6 Mechanical Testing of PLGA/Calcium-Phosphate Cement Composites

For mechanical testing, five PLGA/calcium-phosphate cement composite and five control cylinders were removed from the molds after one day of setting. Subsequently, the cylinders were soaked in Ringer solution (NPBI BV, Emmer-Compascuum, The Netherlands) for three days at 37 °C. Samples were placed in a mechanical testing bench (Instron TT-CM, Instron Corporation, Canton, MA, USA) and compressive strength was measured at a crosshead speed of 1 mm/min.

2.7 Cytotoxicity Assay for Calcium-Phosphate Cement

For the in vitro cytotoxicity assay, the viability of pre-cultured cells was assessed after exposure to conditioned medium of different dilutions (1:1, 1:10, 1:50, 1:100). Three calcium-phosphate cement discs were incubated in 1 ml culture medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum [FBS], 4% L-Glutamine and 0.5% gentamicin) at 37 °C, 95% relative humidity and 5% CO$_2$ in 24 well tissue culture plates. The conditioned medium was removed after 24 h and
diluted 0, 10, 50 and 100 times with unconditioned medium. Concurrently, W20 c17
cells (20th passage, CRC-2623, American Type Culture Collection, Manassas, VA, USA)
were pre-cultured in culture medium on 96 well tissue culture plates at a density of
40,000 cells/cm² (10,000 cells/well) at 37°C, 95% relative humidity, and 5% CO₂. After
twenty-four hours, the culture medium was removed and the cells were exposed to 100
µl of (diluted) conditioned medium at 37°C, 95% relative humidity and 5% CO₂ for 24 h.

After 24 h, the conditioned medium was removed and the cells were rinsed with a
PBS solution twice to remove any remaining conditioned medium. Then, 100 µl
LIVE/DEAD reagent (Molecular Probes Inc., Eugene, OR, USA) were added to each
sample well and incubated in darkness for 30 min at room temperature. The resulting
fluorescence was measured using a fluorescence microplate reader (FLx800, BIO-TEK
Instrument, Winooski, VT, USA). Cells cultured without exposure to conditioned medium
were used as positive control (live) and cells exposed to 70% methanol solution for 30
min served as negative control (dead).

2.8 In Vitro ¹²⁵I-rhBMP-2 Release Kinetics

In vitro release kinetics of ¹²⁵I-rhBMP-2 loaded PLGA microparticles (10 mg of
the unloaded/loaded microparticle mixture [50:50 by weight] per sample) and ¹²⁵I-rhBMP-
2 loaded PLGA/calcium-phosphate cement discs were evaluated. Release kinetics of
rhBMP-2 were studied at pH 7.4 (PBS; Gibco, Grand Island, New York) and pH 4.0 (B-
79 certified buffer, Fisher Scientific, Spring Field, New Jersey). Buffer solutions were
supplemented with 100 µg/ml ampicillin as antibiotic prophylaxis. Samples (n=4 for each
group) were incubated in glass vials containing 3 ml buffer solution at 37°C on a shaker
table (70 rpm) for 28 days. At 1, 4, 7, 14, 21 and 28 days, the buffer solutions were
carefully removed and stored at −20°C until analysis. Subsequently, the samples were
resuspended in fresh buffer. All γ-emission measurements were corrected for radioactive decay by assuming $^{125}$I half-life of 59.4 days.

Statistical Analysis

GraphPad® Instat 3.05 software (GraphPad Software Inc, San Diego, CA, USA) was used for statistical evaluation using an unpaired t-test with Welch correction for the mechanical testing ($p < 0.0001$) and a one-way analysis of variance (ANOVA) with Dunnett multiple comparison post test for the cytotoxicity assay.

3. Results

3.1 Preparation and Characterization of ($^{125}$I rhBMP-2 loaded) PLGA Microparticles

PLGA microparticles were fabricated by a double-emulsion-solvent-extraction technique as previously described [1,6,14]. The average size (and standard deviation) of the microparticle was $17.2 \pm 1.3 \mu m$. Scanning electron microscopy analysis confirmed the spherical shape and the size distribution of the microparticles (Figure 10-1). γ-Counting revealed that the average entrapment efficiency of $^{125}$I rhBMP-2 was 79% ±8%, resulting in a dosage of 0.72 µg rhBMP-2 per mg microparticle.

3.2 Preparation and Characterization of PLGA/Calcium-Phosphate Cement Composite

$^{125}$I-rhBMP-2 loaded PLGA/calcium-phosphate cement discs were prepared according to the described protocol. Discs of 6.35 mm diameter and 2.4 mm thickness had an average weight (and standard deviation) of 76.1 ± 2.6 mg. The average initial $^{125}$I-rhBMP-2 content was 5.0 ± 0.4 µg per average disc. Analysis with micro-computed tomography showed a homogeneous distribution of the microparticles throughout the
scaffold (Figure 10-2). SEM pictures showed that the microparticles maintained their integrity during incorporation into the calcium-phosphate cement (Figure 10-3).

3.3 Mechanical Testing of PLGA/Calcium-Phosphate Cement Composites

Five PLGA/calcium-phosphate cement composite scaffolds and five calcium-phosphate cement scaffolds (controls) were tested in a mechanical testing bench. Results of mechanical testing are depicted in Figure 10-4. Compressive strength was significantly (p<0.001) lower for the PLGA/calcium-phosphate cement composite scaffolds (mean [and standard deviation], 6.4 ± 0.6 MPa) than for the control scaffolds (38.6 ± 2.6 MPa).

3.4 Cytotoxicity Assay Calcium-Phosphate Cement

Results of the cytotoxicity assay are shown in Figure 10-5. Exposure of conditioned medium to pre-cultured W20 c17 cells for 24 h did not significantly affect the viability of the cells compared to exposure of unconditioned medium.

3.5 In Vitro $^{125}$I-rhBMP-2 Release Kinetics

In vitro release kinetics of rhBMP-2 from rhBMP-2 loaded PLGA microparticles (with an average initial loading 3.6 ± 0.0 μg rhBMP-2/sample) and rhBMP-2 loaded PLGA/calcium-phosphate cement discs (with an average initial loading of 5.0 ± 0.4 μg rhBMP-2/disc) were evaluated in two different buffers over the course of 28 days (n=4 for each formulation). All discs maintained their integrity during this period. Normalized cumulative mass release from all samples is depicted in Figure 10-6. None of the formulations displayed a substantial initial burst release. At physiological pH (7.4), the PLGA/calcium-phosphate disks showed a mean initial release of 1.1% ± 0.1% after 24 h, followed by a linear, but marginal release (0.1%/day) resulting in a total cumulative
release of 3.1% ± 0.1% after 28 days. PLGA microparticles showed a mean initial release of 2.4% ± 0.3% after 24 h, followed by a linear release of 0.5% per day. By day 28, a mean of 18.0% ± 1.9% of the total actual loaded rhBMP-2 was released. In an acidic environment (pH 4.0), PLGA/calcium-phosphate discs revealed a greater release of rhBMP-2 after 28 days than PLGA microparticles alone (a mean of 14.5% ± 6.3% compared with a mean of 5.4% ± 0.7%). PLGA microparticles at pH 4.0 showed a mean initial release similar to the release of microparticles at pH 7.4 (2.4% ± 0.5% vs. 2.4% ± 0.3% after 24 h), but release after day 1 was minimal. In the composite group, an increase in the release of rhBMP-2 into the acidic environment was observed from day 21 to day 28. This phenomenon was not observed in the PLGA microparticle group at pH 4.0.

4. Discussion

This study focused on the preparation of an injectable composite material of rhBMP-2 loaded PLGA microparticles and a calcium-phosphate cement. We hypothesized that incorporation of rhBMP-2 loaded microparticles in a calcium-phosphate cement would result in a sustained release of rhBMP-2 under neutral and acidic conditions.

With some PLGA microparticles fabrication techniques, rhBMP-2 is adsorbed on the surface after preparation of the microparticles [15-18]. This method may be advantageous with respect to entrapment efficiency because no proteins are lost during the fabrication process. On the other hand, it does not provide encapsulation of proteins, which would allow for greater control of the release kinetics of the rhBMP-2. It seems less suitable for our PLGA/calcium-phosphate composite as the high protein concentration present at the outer surface of the microparticles might interfere with the setting reaction of the cement [19]. Therefore, PLGA microparticles were produced and
loaded with rhBMP-2 by an established technique which produced microparticles with similar dimensions as reported in previous papers [1,2,6,20].

Pilot studies have confirmed that microparticles can indeed be incorporated into a calcium-phosphate cement at various PLGA/calcium-phosphate ratios up to 50/50 by weight. For this study, an intermediate PLGA/calcium-phosphate ratio of 30/70 by weight was chosen. Mechanical testing of this formulation showed that this composite was significantly (p < 0.001) weaker than calcium-phosphate cement without PLGA microparticles, but it was stronger than previously described macroporous calcium-phosphate cement [7]. Whereas the cytotoxicity of PLGA has already been investigated thoroughly, this study demonstrated that the calcium-phosphate cement exhibits no cytotoxic effects.

A dosage of 5 μg rhBMP-2 per 75 mm³ scaffold has been shown to be within the therapeutical range of this growth factor in rats and rabbits [12,13]. We achieved this dosage with a 50/50 by weight ratio of rhBMP-2 loaded/unloaded microparticles. Consequently, the dosage of rhBMP-2 within the scaffold can be adjusted by altering the loaded to unloaded microparticle ratio without altering the composite properties.

The entrapment efficiency of rhBMP-2 in PLGA microparticles was a mean (and standard deviation) of 79% ± 8% was achieved. This percentage is within the range of previously published studies that varied in entrapment between 37% and 91% [1,3,6,21]. It must be emphasized however, that drug properties, drug concentration, and polymer composition play an important role in entrapment efficiency [1,6]. In the current study, the influence of these parameters upon the entrapment of rhBMP-2 into PLGA microparticles was not explored. The formulation used was chosen on the basis of the results of previous studies. For example, in order to minimize rhBMP-2 loss, poly(ethylene glycol) (PEG) was not added as it has been shown that the addition of
PEG negatively influences the entrapment efficiency of TGF-β1 in PLGA microparticles fabricated with the same method [1].

The release kinetics of our composite in two buffer solutions i.e. neutral (pH 7.4) and acidic (pH 4.0) were investigated. An acidic pH buffer was used to simulate inflammatory phase of wound healing and degradation of the polymer. In addition, the release kinetics of rhBMP-2 loaded PLGA microparticles alone was determined for use as a control. Release of rhBMP-2 was very limited for all formulations. In the PLGA microparticle control group, a mean of 18.0% ± 1.9% of the rhBMP-2 was released after 28 days. This release is marginal compared to previous studies under similar, but in some possibly important respects, different conditions. For example, Lu et al found a cumulative release from PLGA microparticles of 68% (loading density 6.0 ng TGF-β1/mg PLGA) after 28 days with the co-encapsulation of FITC-BSA (loading density 4 μg/mg PLGA) [1]. Peter et al determined a cumulative TGF-β1 release of 33% (loading density 6.0 ng TGF-β1/mg PLGA/PEG blend with 0.5% PEG) after 28 days under co-encapsulation of FITC-dextran (loading density 10 μg/mg PLGA/PEG) [6]. Hedberg et al showed a cumulative release of 62% (loading density 204 μg TP 508/mg PLGA) after 28 days without co-encapsulation, but the bioactive molecule released was very small (23 amino acid peptide) compared with rhBMP-2 and TGF-β1 [2]. In the current study, PBS/BSA [0.1%] buffer was used as dilution agent for rhBMP-2 solution as recommended by the manufacturer of rhBMP-2. The amount of co-encapsulated BSA was minimal compared to the amount of co-encapsulated molecules in the studies of Lu et al and Peter et al. Therefore, we hypothesize that the amount of co-encapsulated protein may influence the final rhBMP-2 release profile.

The composite material revealed a mean release of 3.1% ± 0.1% after 28 days under neutral conditions. The difference with the PLGA microparticles control group (mean, 18.0% ± 1.9%) was significant. The physical entrapment of microparticles within
the nanoapatitic porous cement [22] may have contributed to the limited release of rhBMP-2 from the PLGA/calcium-phosphate composites. A high binding affinity for proteins exhibited by ceramics [11] may have diminished the amount of rhBMP-2 released from the composite. After release from the PLGA microparticles, rhBMP-2 may have bound to the calcium-phosphate cement resulting in delayed release from the composite. Thus, the nanoporosity of the calcium-phosphate cement not only did not facilitate the release of rhBMP-2 but may have further limited it due to the protein adsorption on the ceramic.

An acidic environment increased the release of rhBMP-2 from the composite material. However, release in the PLGA microparticle control group was decreased compared to neutral conditions. Therefore, it appears unlikely that PLGA in the composite plays a role in the observed increase of release. On the contrary, it is known that calcium-phosphate cement dissolves in an acidic environment. Consequently, calcium-phosphate cement bound rhBMP-2 might be released after dissolving of the calcium-phosphate cement. This phenomenon also supports our earlier suggestion that rhBMP-2 binds to the surrounding calcium-phosphate immediately after release from the PLGA due to the high binding affinity of proteins for calcium-phosphate cement.

5. Conclusions

This study demonstrated the feasibility of fabrication of an injectable calcium phosphate cement embedded with biodegradable polymeric microparticles loaded with rhBMP-2. The results show that the composite composition and nanostructure as well as the environmental pH influence rhBMP-2 release from the composites. The data further suggest that the affinity of the protein to the ceramic carrier influences the amount of protein released and factors altering the dissolution of the ceramic carrier
such as pH affect the release kinetics. Finally, this study demonstrates that the calcium-phosphate cement exhibits no cytotoxic effects.
**Figure 10-1:** Scanning electron micrograph of PLGA microparticles, showing the spherical shape of the microparticles. Bar represents 10 μm.
Figure 10-2: Micro-computed tomography of 30/70 wt% PLGA/Ca-P cement composite. In the μCT reconstruction, PLGA microparticles appear as pores in the Ca-P cement. Bar represents 1 mm.
Figure 10-3: Scanning electron micrograph of 30/70 wt% PLGA/Ca-P cement composite. Several different sized microparticles surrounded by microporous Ca-P cement can be observed. Bar represents 20 μm.
Figure 10-4: Results of mechanical testing of Ca-P cement and PLGA/Ca-P cement composite. Error bars represent means ± standard deviation for n=5. The asterisk indicates a significant difference between both groups (p<0.001).
**Figure 10-5:** Results of LIVE-DEAD cytotoxicity assay of Ca-P cement. Normalized viability, based on total fluorescence, is displayed for cells exposed to unconditioned medium (control) and cells exposed to conditioned medium in different dilutions. Error bars represent means ± standard deviation for n=3. Variation among LIVE bar means is not significantly greater than expected by chance (p > 0.05).
Figure 10-6: In vitro release of rhBMP-2 loaded PLGA microparticles and PLGA/Ca-P cement composite in pH 7.4 and pH 4.0 under continuous shaking at 37°C. Error bars represent means ± standard deviation for n=4.
CHAPTER XII

REFERENCES

Chapter I


Chapter III


Chapter IV


Chapter V


Chapter VI


Chapter VII


Chapter VIII


Chapter IX


Chapter X


