Designing a Whey Protein Based Material as a Scaffold for Bone Regeneration

Mia Dvora
Louisiana State University and Agricultural and Mechanical College

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DESIGNING A WHEY PROTEIN BASED MATERIAL AS A SCAFFOLD FOR BONE REGENERATION

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Gordon A. and Mary Cain Department of Chemical Engineering

by
Mia Dvora
B.S., University of Florida, 2005
August 2010
To my parents Debbie and Yair

and to Hila, Nina, and Etai
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# Table of Contents

Acknowledgements.............................................................................................................. iii  
List of Tables ......................................................................................................................... viii  
List of Figures ....................................................................................................................... ix  
Abstract ................................................................................................................................ xvi

Chapter 1: Introduction and Background............................................................................ 1  
1.1. Context ......................................................................................................................... 1  
1.2. Motivation .................................................................................................................... 1  
1.2.1. Bone Grafts ............................................................................................................. 2  
1.2.2. Metals and Ceramics in Bone Replacement ........................................................... 2  
1.2.3. Polymers as an Alternative .................................................................................... 3  
1.2.4. Characterization Void for Whey Protein ................................................................. 5  
1.3. Rationale ..................................................................................................................... 5  
1.3.1. Therapeutic Scheme .............................................................................................. 5  
1.3.2. Material Selection ................................................................................................. 6  
1.4. Goals and Objectives .................................................................................................. 7  
1.5. References .................................................................................................................. 8

Chapter 2: Assessing the Mechanical Properties and Biocompatibility of Whey Protein Isolate Gels Toward Use in Bone Regeneration...................................................... 13  
2.1. Introduction ................................................................................................................. 13  
2.2. Materials and Methods ............................................................................................... 15  
2.2.1. Materials ................................................................................................................ 15  
2.2.2. Scaffold Fabrication .............................................................................................. 16  
2.2.3. Ultimate Mechanical Testing ............................................................................ 17  
2.2.4. In Vitro Testing ................................................................................................... 17  
2.2.5. Scanning Electron Microscopy ........................................................................... 18  
2.2.6. Statistical Analysis .............................................................................................. 18  
2.3. Results and Discussion .............................................................................................. 18  
2.3.1. Concentration Effects of Whey Protein Isolate .................................................... 18  
2.3.2. Concentration Effects of Calcium Chloride ........................................................ 23  
2.3.3. In Vitro Scaffold Performance .......................................................................... 28  
2.4. Conclusions ............................................................................................................... 30  
2.5. References ................................................................................................................. 30

Chapter 3: Measuring Hydration Characteristics of High-Concentration Whey Protein Isolate Hydrogels.................................................................................................................. 34  
3.1. Introduction ............................................................................................................... 34  
3.2. Materials and Methods .............................................................................................. 35
Chapter 5: Proliferation Kinetics and Mineralization Properties of MC3T3-E1 Cells on Whey Protein Isolate Scaffolds for Bone Tissue Regeneration

5.1. Introduction ........................................................................................................... 60
5.2. Materials and Methods ......................................................................................... 62
  5.2.1. Materials ......................................................................................................... 62
  5.2.2. Scaffold Fabrication and Processing ................................................................. 62
     5.2.3. In Vitro Culture ............................................................................................. 63
  5.2.4. Mineralization Studies ..................................................................................... 63
  5.2.5. Fluorescence Microscopy .............................................................................. 63
  5.2.6. Curve Fitting ................................................................................................ 64
  5.2.7. Scanning Electron Microscopy with Energy Dispersive X-Ray Spectroscopy .. 65
      5.2.8. Statistical Analysis ....................................................................................... 66
5.3. Results and Discussion ......................................................................................... 66
  5.3.1. Direct Cell Count ............................................................................................ 66
  5.3.2. Non-Linear Regression .................................................................................. 67
  5.3.3. Seeding Efficiency ......................................................................................... 67
  5.3.4. Exponential Growth Rate Constant ............................................................... 71
  5.3.5. Gompertz Function Rate Constants ............................................................. 72
  5.3.6. Scaffold Mineralization ................................................................................. 74
5.4. Conclusions ........................................................................................................... 79
5.5. References ............................................................................................................ 81
Chapter 6: Pore Structure Analysis of Whey Protein Isolate Gels by X-Ray Microcomputed Tomography ................................................................. 84
  6.1. Introduction .......................................................................................... 84
  6.2. Materials and Methods ........................................................................ 85
       6.2.1. Materials ..................................................................................... 85
       6.2.2. Sample Fabrication ...................................................................... 86
       6.2.3. Microtomography ......................................................................... 86
       6.2.4. Network Modeling ....................................................................... 88
       6.2.5. Viscometry .................................................................................. 91
       6.2.6. Theoretical Threshold Viscosity .................................................... 91
  6.3. Results .................................................................................................. 92
       6.3.1. Porosity ....................................................................................... 92
       6.3.2. Interconnectivity .......................................................................... 92
       6.3.3. Pore and Throat Diameters ......................................................... 93
       6.3.4. Viscometry ................................................................................ 96
       6.3.5. Theoretical Threshold Viscosity .................................................... 96
  6.4. Discussion ............................................................................................ 97
  6.5. Conclusions ........................................................................................ 100
  6.6. References .......................................................................................... 101

Chapter 7: Method for Electrospinning Pure Whey Protein Isolate and Characterization of Electrospun Films .............................................................. 105
  7.1. Introduction .......................................................................................... 105
  7.2. Materials and Methods ........................................................................ 107
       7.2.1. Materials ..................................................................................... 107
       7.2.2. Electrospinning ........................................................................... 107
       7.2.3. Fiber Characterization ................................................................. 108
  7.3. Results .................................................................................................. 108
       7.3.1. Concentration Effects .................................................................. 108
       7.3.2. Flow Rate Effects ........................................................................ 109
       7.3.3. Voltage Effects ............................................................................ 110
       7.3.4. Collector Distance Effects ........................................................... 111
  7.4. Discussion ............................................................................................ 111
  7.5. Conclusions ........................................................................................ 113
  7.6. References .......................................................................................... 113

Chapter 8: Conclusions, Optimization, and Future Outlook ................................ 115
  8.1. Conclusions ........................................................................................ 115
       8.1.1. Mechanical Properties of the Naïve Gel ....................................... 115
       8.1.2. Hydrogel Swelling Properties ....................................................... 115
       8.1.3. Mechanical Properties of Reinforced Composites ....................... 116
       8.1.4. Biocompatibility and Cell-Matrix Interactions ............................. 117
       8.1.5. Pore Network Structure of Naïve Gels .......................................... 118
       8.1.6. WPI Electrospinning ................................................................. 119
  8.2. Scaffold Optimization ........................................................................... 119
  8.3. Future Paths ......................................................................................... 120
Appendix A: Cure-Time Profiles for Whey Protein Isolate Gels ................................................. 125
Appendix B: Scanning Electron Micrographs of MC3T3-E1 Cells Adhered to a Whey Protein Isolate Surface ........................................................................................................... 126
Appendix C: Detailed Results for Swelling Studies to Supplement Chapter 3 .................... 131
  C.1. Individual-Gel Swelling Curves .................................................................................... 131
  C.2. SAS Regression and Output ......................................................................................... 131
    C.2.1. Output for Mass Change with respect to WPI ......................................................... 138
    C.2.2. Output for Volume Change with respect to WPI ...................................................... 143
    C.2.3. Output for Mass Change with respect to CaCl$_2$ .................................................... 148
    C.2.4. Output for Volume Change with respect to CaCl$_2$ ................................................ 153
Appendix D: Supporting Material for Proliferation and Mineralization of MC3T3-E1 Cells on Whey Protein Isolate Scaffolds ................................................................. 159
Appendix E: Morphology and Internal Structure of MC3T3-E1 Cells on Whey Protein Isolate Scaffolds ........................................................................................................ 161
  E.1. Background .................................................................................................................. 161
  E.2. Methods ....................................................................................................................... 161
  E.3. Results ......................................................................................................................... 162
Appendix F: Supporting Material for Pore Network Characterization of Whey Protein Isolate Gels ........................................................................................................ 164
  F.1. Axial Profiles of Pore Network Characteristics .......................................................... 164
  F.2. Critical Viscosity Calculation ..................................................................................... 165
    F.2.1. Theory .................................................................................................................... 165
    F.2.2. Calculation Assumptions ....................................................................................... 165
    F.2.3. Variable Definitions .............................................................................................. 166
    F.2.4. Force Balance ....................................................................................................... 167
    F.2.5. Drag Force Expression ......................................................................................... 167
    F.2.6. Threshold Viscosity from Force Balance ............................................................. 168
  F.3. References .................................................................................................................. 169

Vita .......................................................................................................................................... 170

vii
List of Tables

Table 5.1 – Non-Linear Regression Parameters Describing Osteoblastic Proliferation on WPI Scaffolds. ........................................................................................................................................................................... 69
List of Figures

Fig. 2.1 – Mechanical strength versus WPI concentration. The curves represent stress at failure under compression and tension of WPI gels of 10 mM CaCl$_2$. Trend lines, consistent with statistical findings, are meant to guide the eye. ................................................................. 19

Fig. 2.2 – Modulus of elasticity versus WPI concentration. The lines represent compressive modulus and Young’s modulus of WPI gels of 10 mM CaCl$_2$. Trend lines represent linear fits. 19

Fig. 2.3 – Scanning electron micrographs of WPI gels of (a) 20% w/v WPI, (b) 35% w/v WPI, and (c) 45% w/v WPI. Higher magnification of (a), (b), and (c) is shown in micrographs (d), (e), and (f), respectively. The SEMs demonstrate the effect of WPI concentration on the microarchitecture of the gels. Scales across each row are identical. ......................................................... 20

Fig. 2.4 – Mechanical strength vs. CaCl$_2$ concentration. The curves represent stress at failure under compression of 30% w/v WPI and 35% WPI, and under tension of WPI gels of 30% w/v WPI and 40% w/v WPI. Inlay plot provides a clearer representation of the tensile results. Trend lines, consistent with statistical findings, are meant to guide the eye................................................................. 24

Fig. 2.5 – Modulus of elasticity vs. CaCl$_2$ concentration. The curves represent Young’s modulus of gels of 30% w/v WPI and 40% WPI, and compressive modulus of gels of 30% w/v WPI and 35% WPI. Trend lines, consistent with statistical findings, are meant to guide the eye. ............. 24

Fig. 2.6 – Scanning electron micrographs of WPI gels of 30% w/v WPI and (a) 0 mM CaCl$_2$, (b) 10 mM CaCl$_2$, and (c) 40 mM CaCl$_2$. Higher magnification of (a), (b), and (c) is shown in micrographs labeled (d), (e), and (f), respectively. The SEMs demonstrate the effect of CaCl$_2$ concentration on the microarchitecture of the gels. Scales across each row are identical.......... 26

Fig. 2.7 – Scanning electron micrographs of WPI gels of 40% w/v WPI and (a) 0 mM CaCl$_2$, (b) 10 mM CaCl$_2$, and (c) 40 mM CaCl$_2$. Higher magnification of (a), (b), and (c) is shown in micrographs (d), (e), and (f), respectively. The SEMs demonstrate the effect of CaCl$_2$ concentration on the microarchitecture of the gels. Scales across each row are identical........ 27

Fig. 2.8 – Scanning electron micrographs of MC3T3-E1 cells cultured on a WPI gel surface. The desired flat, stellate morphology is that observed for the imaged cells. ......................................................... 28

Fig. 3.1 – Initial water content in the fresh gels as a function of WPI concentration. Water content is shown as (a) mass loss, and (b) volume loss upon drying. Data are presented as percent loss of original mass or volume of each gel; error bars represent standard deviation. ..................... 37

Fig. 3.2 – Comparison of exponent $b$ from power law regression for the varying (a) WPI concentrations and (b) CaCl$_2$ concentrations. Error bars represent 95% confidence intervals on the parameters. ........................................................................... 38
Fig. 3.3 – Gel swelling curves at varying WPI concentrations. Water uptake in terms of (a) increased mass and (b) increased volume of gels with respect to time. Expanded views of short times for (a) and (b) are presented in (c) and (d), respectively. Data are represented as percent increase over initial mass or volume; curves represent power-law fits to the data. 39

Fig. 3.4 – Gel swelling curves at varying CaCl₂ concentrations. Water uptake in terms of (a) increased mass and (b) increased volume of gels with respect to time. Expanded views of short times for (a) and (b) are presented in (c) and (d), respectively. Data are represented as percent increase over initial mass or volume; curves represent power-law fits to the data. 41

Fig. 4.1 – Molecular structures of polysaccharide additives. (a) Amylose, or amylopectin where amylopectin also contains α(1-6) branches; (b) cellulose; (c) chitosan; (d) dextran. 49

Fig. 4.2 – Composite compressive strength vs. ratio of polysaccharide to WPI. All composites contain 35% w/v WPI and 10 mM CaCl₂. Nominal dotted line represents average naïve WPI gel strength. 50

Fig. 4.3 – Composite compressive modulus vs. ratio of polysaccharide to WPI. All composites contain 35% w/v WPI and 10 mM CaCl₂. Nominal dotted line represents average naïve WPI gel modulus. 50

Fig. 4.4 – Composite break strain vs. ratio of polysaccharide to WPI. All composites contain 35% w/v WPI and 10 mM CaCl₂. Nominal dotted line represents average naïve WPI gel break-strain. 50

Fig. 4.5 – Compressive strength (a) and compressive modulus (b) of amylose- or amylopectin-based composites. Composites contain 35% w/v WPI and 10 mM CaCl₂. 52

Fig. 4.6 – Compressive strength (a) and modulus (b) of amylopectin-based composite compared to naïve WPI gels. Composites contain 10 mM CaCl₂ and 0.77 g amylopectin per g WPI – corresponding to 0.2 g amylopectin per g WPI for 35% w/v WPI composite. Symbols correspond to difference between composite and corresponding naïve gel of significance of p < 0.05 (*), p < 0.01 (†), p < 0.005 (**), p < 0.001 (‡), or p < 0.0005 (‡‡). 53

Fig. 5.1 – Exponential growth curves for varying scaffold WPI concentration. Proliferation data for MC3T3-E1, subclone 4 cells cultured for 1-14 days on scaffolds containing 10 mM CaCl₂ and 0% amylopectin and varying in WPI concentration. Curves represent regressed fits of the data to the exponential growth model. 68

Fig. 5.2 – Exponential growth curves for varying scaffold CaCl₂ concentration. Proliferation data for MC3T3-E1, subclone 4 cells cultured for 1-14 days on scaffolds containing 35% w/v WPI and 0% amylopectin and varying in CaCl₂ concentration. Curves represent regressed fits of the data to the exponential growth model. 68

Fig. 5.3 – Exponential growth curves for varying scaffold amylopectin concentration. Proliferation data for MC3T3-E1, subclone 4 cells cultured for 1-14 days on scaffolds containing 35% w/v WPI and 10 mM CaCl₂ and varying in amylopectin concentration. Curves represent regressed fits of the data to the exponential growth model. 70
Fig. 5.4 – Gompertz model growth curves for varying scaffold WPI concentration. Proliferation data for MC3T3-E1, subclone 4 cells cultured for 1-21 days on scaffolds containing 10 mM CaCl$_2$ and 0% amylopectin and varying in WPI concentration. Curves represent regressed fits of the data to the Gompertz growth model.

Fig. 5.5 – Gompertz model growth curves for varying scaffold CaCl$_2$ concentration. Proliferation data for MC3T3-E1, subclone 4 cells cultured for 1-21 days on scaffolds containing 35% w/v WPI and 0% amylopectin and varying in CaCl$_2$ concentration. Curves represent regressed fits of the data to the Gompertz growth model. Dotted lines represent the best fit for data sets with unstable solutions.

Fig. 5.6 – Gompertz model growth curves for varying scaffold amylopectin concentration. Proliferation data for MC3T3-E1, subclone 4 cells cultured for 1-21 days on scaffolds containing 35% w/v WPI and 10 mM CaCl$_2$ and varying in amylopectin concentration. Curves represent regressed fits of the data to the Gompertz growth model.

Fig. 5.7 – SEM micrographs and elemental analysis of mineralized scaffolds. SEM micrographs of (a) MC3T3-E1, subclone 4 cells on a scaffold containing 35% w/v WPI scaffolds, (b) acellular scaffold containing 45% w/v WPI, and (c) subclone 24 (non-differentiating) cells on a scaffold containing 20% w/v WPI – all cultured for 28 days in mineralization medium. Scale bar = 20 μm; inlay bar = 2 μm. EDS spectra with elemental analysis of (a), (b), and (c) are presented in (d), (e), and (f), respectively. X axis represents energy in keV; y axis represents intensity in counts.

Fig. 5.8 – SEM micrographs and elemental analysis of mineralized scaffolds of varying CaCl$_2$ concentrations. SEM micrographs of 35% w/v WPI scaffolds (a) containing 0 mM CaCl$_2$ with MC3T3-E1, subclone 4 (differentiating) cells, (b) acellular, containing 0 mM CaCl$_2$, (c) containing 20 mM CaCl$_2$ with subclone 4 cells, and (d) acellular, containing 20 mM CaCl$_2$ – all cultured for 28 days in mineralization medium. Scale bar = 50 μm; inlay bar = 2 μm. EDS spectra with elemental analysis of (a), (b), (c), and (d) are presented in (e), (f), (g), and (h), respectively. X axis represents energy in keV; y axis represents intensity in counts.

Fig. 5.9 – SEM micrographs and elemental analysis of mineralized scaffolds of varying amylopectin concentration. SEM micrographs of 35% w/v WPI and 10 mM CaCl$_2$ scaffolds (a) containing 0.05 g amylopectin per g WPI with MC3T3-E1, subclone 4 cells, (b) acellular, containing 0 g amylopectin per g WPI, (c) containing 0.25 g amylopectin per g WPI with subclone 4 (differentiating) cells, and (d) containing 0.25 g amylopectin per g WPI with subclone 24 (non-differentiating) cells – all cultured for 28 days in mineralization medium. Scale bar = 50 μm; inlay bar = 5 μm. EDS spectra with elemental analysis of (a), (b), (c), and (d) are presented in (e), (f), (g), and (h), respectively. X axis represents energy in keV; y axis represents intensity in counts.

Fig. 6.1 – Preliminary data forms of XMCT data. (a) 2D slice of 45% w/v WPI with grayscale values indicated, and (b) the associated histogram of a 16-bit grayscale image for which the binary thresholding values of 6690 and 6710 were chosen.

Fig. 6.2 - 3D rendered images of (a) 20%, (b) 35%, (c) 45% w/v WPI gels, and (d-f) their respective ball-and-stick pore network representations.
Fig. 6.3 – Mean bulk values of (a) porosity, (b) pore coordination number, (c) pore diameter, and (d) throat diameter for 20%, 35%, and 45% w/v WPI gels as obtained by analysis of XMCT data. ................................................................. 93

Fig. 6.4 – Radial profiles of (a) porosity, (b) pore coordination number (c) pore diameter and (d) throat diameter for 20% (blue), 35% (red), and 45% (green) w/v WPI gels as obtained by analysis of XMCT data. ................................................................. 94

Fig. 6.5 – Pore size distributions of 20%, 35%, and 45% w/v WPI gels as determined by analysis of XMCT data. .................................................................................................................. 95

Fig. 6.6 – Viscosity variation with WPI suspension concentration. (a) Viscosities of WPI gel precursor suspensions measured over a range of shear rates. (b) Viscosities for a shear rate of 10.5 s^{-1} using cone and plate configuration; the calculated threshold viscosity to support 100 μm bubbles is indicated by the horizontal line. ................................................................. 97

Fig. 7.1 – Electrosprining Setup. (a) Process schematic depicting the solution in the syringe, flowing through the needle, subjected to a voltage and spinning onto a grounded collection plate; (b) Photograph of custom-designed electrosprining apparatus used in this study. ............... 106

Fig. 7.2 – Fiber morphology depends on processing parameters. (a) Beading effect and heterogeneous fibers, (b) thick, uniform fibers in interconnected network, and (c) thin, smooth, uniform fibers. Scale bar = 20 μm. ................................................................................................. 108

Fig. 7.3 – Dependence of electrosprined fiber diameter on protein concentration. Empty markers represent non-spinning solutions. ........................................................................................................... 109

Fig. 7.4 – Dependence of electrosprined fiber diameter on solution flow rate. ......................... 109

Fig. 7.5 – Dependence of electrosprined fiber diameter on applied voltage. Empty marker indicates non-spinning voltage. ................................................................................................. 110

Fig. 7.6 – WPI fibers electrosprined at (a) 12.5 kV, (b) 17.5 kV, (c) 20.0 kV, (d) 22.5 kV, and (e) 27.5 kV ................................................................................................................................. 110

Fig. 7.7 – Dependence of electrosprined fibers on collector plate distance from the needle tip. Empty marker indicates non-spinning distance. ........................................................................................................ 111

Fig. A.1 – Mechanical properties of WPI gels as a function of cure time. Plots showing (a) compressive strength, (b) compressive modulus, and (c) break strain as functions of cure time. The results led to the selection of 60 minutes as the optimal cure time for compression samples of WPI gels (radius = 5 mm). ................................................................. 125

Fig. B.1 – SEM micrograph depicting cells adhered to WPI surface. ........................................ 126

Fig. B.2 – SEM micrograph depicting cells adhered to WPI surface. ........................................ 126

Fig. B.3 – SEM micrograph depicting cells adhered to WPI surface. ........................................ 127
Fig. B.4 – SEM micrograph depicting cells adhered to WPI surface. ........................................ 127
Fig. B.5 – SEM micrograph depicting cells adhered to WPI surface. ........................................ 127
Fig. B.6 – SEM micrograph depicting cells adhered to WPI surface. ........................................ 127
Fig. B.7 – SEM micrograph depicting cells adhered to WPI surface. ........................................ 127
Fig. B.8 – SEM micrograph depicting cells adhered to WPI surface. ........................................ 127
Fig. B.9 – SEM micrograph depicting cells adhered to WPI surface. ........................................ 128
Fig. B.10 – SEM micrograph depicting cells adhered to WPI surface. ....................................... 128
Fig. B.11 – SEM micrograph depicting cells adhered to WPI surface. ....................................... 128
Fig. B.12 – SEM micrograph depicting cells adhered to WPI surface. ....................................... 128
Fig. B.13 – SEM micrograph depicting cells adhered to WPI surface. ....................................... 128
Fig. B.14 – SEM micrograph depicting cells adhered to WPI surface. ....................................... 128
Fig. B.15 – SEM micrograph depicting cells adhered to WPI surface. ....................................... 129
Fig. B.16 – SEM micrograph depicting cells adhered to WPI surface. ....................................... 129
Fig. B.17 – SEM micrograph depicting cells adhered to WPI surface. ....................................... 129
Fig. B.18 – SEM micrograph depicting cells adhered to WPI surface. ....................................... 129
Fig. B.19 – SEM micrograph depicting cells adhered to WPI surface. ....................................... 129
Fig. B.20 – SEM micrograph depicting cells adhered to WPI surface. ....................................... 129
Fig. B.21 – SEM micrograph depicting cells adhered to WPI surface. ....................................... 130
Fig. B.22 – SEM micrograph depicting cells adhered to WPI surface. ....................................... 130
Fig. B.23 – SEM micrograph depicting cells adhered to WPI surface. ....................................... 130
Fig. B.24 – SEM micrograph depicting cells adhered to WPI surface. ....................................... 130
Fig. B.25 – SEM micrograph depicting cells adhered to WPI surface. ....................................... 130

Fig. C.1 – Swelling curves for gels composed of 20% w/v WPI and 10 mM CaCl₂. (a) Swelling defined by mass increase; (b) swelling defined by volume increase. ................................. 131

Fig. C.2 – Swelling curves for gels composed of 25% w/v WPI and 10 mM CaCl₂. (a) Swelling defined by mass increase; (b) swelling defined by volume increase. ................................. 132
Fig. C.3 – Swelling curves for gels composed of 30% w/v WPI and 10 mM CaCl$_2$. (a) Swelling defined by mass increase; (b) swelling defined by volume increase. .................................................. 132

Fig. C.4 – Swelling curves for gels composed of 35% w/v WPI and 10 mM CaCl$_2$. (a) Swelling defined by mass increase; (b) swelling defined by volume increase. .................................................. 133

Fig. C.5 – Swelling curves for gels composed of 40% w/v WPI and 10 mM CaCl$_2$. (a) Swelling defined by mass increase; (b) swelling defined by volume increase. .................................................. 133

Fig. C.6 – Swelling curves for gels composed of 45% w/v WPI and 10 mM CaCl$_2$. (a) Swelling defined by mass increase; (b) swelling defined by volume increase. .................................................. 134

Fig. C.7 – Swelling curves for gels composed of 35% w/v WPI and 0 mM CaCl$_2$. (a) Swelling defined by mass increase; (b) swelling defined by volume increase. .................................................. 134

Fig. C.8 – Swelling curves for gels composed of 35% w/v WPI and 5 mM CaCl$_2$. (a) Swelling defined by mass increase; (b) swelling defined by volume increase. .................................................. 135

Fig. C.9 – Swelling curves for gels composed of 35% w/v WPI and 10 mM CaCl$_2$. (a) Swelling defined by mass increase; (b) swelling defined by volume increase. .................................................. 135

Fig. C.10 – Swelling curves for gels composed of 35% w/v WPI and 20 mM CaCl$_2$. (a) Swelling defined by mass increase; (b) swelling defined by volume increase. .................................................. 136

Fig. C.11 – Swelling curves for gels composed of 35% w/v WPI and 35 mM CaCl$_2$. (a) Swelling defined by mass increase; (b) swelling defined by volume increase. .................................................. 136

Fig. C.12 – Swelling curves for gels composed of 35% w/v WPI and 50 mM CaCl$_2$. (a) Swelling defined by mass increase; (b) swelling defined by volume increase. .................................................. 137

Fig. C.13 – Sample program code for swelling-curve regressions. This code was used for all fits, with data file names and variables as the only changes between runs............................................... 137

Fig. D.1 – Representative scaffold surface used in direct cell count. Every scaffold (shown: 40% WPI; 10 mM CaCl$_2$; 0% amylopectin), after a prescribed incubation, was fixed, permeabilized, and stained with Hoechst 33342 nuclear dye. In this representative image, the cell nuclei are seen as bright blue spots. Fourteen such images were captured per repeat, and at least three repeats were used per data point. ........................................................................................................ 159

Fig. D.2 – Exponential growth curves for CaCl$_2$ concentrations unsuitable for Gompertz function regression. Proliferation data for MC3T3-E1, subclone 4 cells cultured for 1-21 days on scaffolds containing 35% w/v WPI and 0% amylopectin and 5 or 15 mM CaCl$_2$. Data points represent averages of at least 3 replications; curves represent regressed fits of the data to the exponential growth model. As a result of the regression: SE = 1.48; k = 0.152 for 5 mM CaCl$_2$, SE = 0.787; k = 0.190. ........................................................................................................ 159

Fig. D.3 – SEM micrograph and elemental analysis of mineralized scaffold containing 20 mM CaCl$_2$. The scaffold represented in the micrograph contains 35% w/v WPI and 20 mM CaCl$_2$, 

xiv
and was cultured with MC3T3-E1, subclone 4 (differentiating) cells for 28 days in mineralization medium. Scale bar = 20 μm. EDS spectrum corresponds to a point scan on the background surface of the scaffold. The spectrum contains proof of mineralized content on the scaffold surface. ................................................................. 160

Fig. D.4 – Sample SAS program for non-linear regression. A variation of this code was used for all non-linear regressions in Chapter 5 and Figure D.2 ................................................................. 160

Fig. E.1 – Phalloidin-stained actin filaments (red) by confocal microscopy. The cytoskeleton of MC3T3-E1 cells adhered to WPI gel scaffolds is labeled to visualize the morphology of the cells growing on (a, b) 20% w/v WPI gels and (c, d) 45% w/v WPI gels. ........................................... 162

Fig. E.2 – Example of cell exhibiting a poorly-organized cytoskeleton. The stained actin is delocalized instead of being drawn in tight filaments. This morphology indicates a distressed cell. Few such cells were observed on the surfaces of WPI scaffolds ......................................................... 163

Fig. F.1 – Axial profiles of (a) porosity, (b) pore coordination number, (c) pore diameter, and (d) throat diameter for 20% (blue), 35% (red), and 45% (green) w/v WPI gels as obtained by analysis of XMCT data. .................................................................................. 164

Fig. F.2 – Force balance on a bubble in an infinite WPI suspension. F_B, F_g, and F_D that are acting on the bubble represent the buoyant force, gravitational force, and drag force, respectively. ... 167
Abstract

A novel gel material was designed and optimized for use as a bone tissue regeneration scaffold. Whey protein isolate (WPI), the primary component of the material, underwent considerable testing for conformity to a set of known material characteristics required for application in bone regeneration. WPI gels of different compositions were fabricated by thermally inducing gelation of high-concentration protein suspensions, and characterized for compressive strength and modulus, hydration swelling and drying properties, mechanical behavior change due to polysaccharide additives, and intrinsic pore network structure. The gels were also tested for their compatibility with MC3T3-E1 cells, and interactions such as cell adhesion, cytotoxicity, proliferation kinetics, and bone formation, were characterized for gels of different compositions. Some properties of interest were composition- and processing-dependent, while others varied little with such variables.

Results revealed that the most favorable mechanical properties could be obtained by using a material of 40% w/v WPI, 10 mM CaCl₂, and 0.2 g amylopectin per g WPI. The mechanical properties of this composite approached the ultimate strength necessary for a load-bearing scaffold, and were within one order of magnitude of the lower limit of the necessary compressive modulus. The proper modulus could likely be achieved by converting the conventional composite to a nanocomposite.

The observed cell-scaffold interactions were highly suitable. All tested naïve gels and composites supported the adhesion and proliferation of the model cell line for extended culture periods. Amylopectin incorporation decreased initial preosteoblast adhesion but improved the
proliferation rate constant – the more important system parameter. Both the naïve gel and the composites enabled cells to differentiate and create bone in vitro, and sustained viability for the length of the 4-week study.

The current fabrication technique left insufficient porosity and interconnectivity for a bone scaffold, though the necessary pore size distribution was achieved. The effect of WPI concentration and precursor suspension viscosity on these properties was thoroughly characterized. In order to correct the disparity in properties, a method for electrospinning WPI was developed, and shows great promise. While further studies are required, the developed composite has significant potential for implementation in the industry.
Chapter 1: Introduction and Background

1.1. Context
The field of tissue engineering and regenerative medicine is making strides in succeeding the field of organ and tissue replacement for the improved safety and affordability of medical care and the increase of patient lifespan and improvement of quality of life. In the midst of advancement in the field, many hurdles remain in the path to implementation of the techniques developed for regenerating bone following massive tissue loss caused by injury or disease. In this project, whey protein isolate (WPI) is presented as a promising material upon which to base scaffold design for application in the field of tissue regeneration.

1.2. Motivation
As life expectancy in the United States and all over the world steadily rises, so does the necessity for viable tissue regeneration techniques to address the needs of the ever-increasing aging population. In 2005, $17 billion were incurred in direct healthcare costs from osteoporotic-related fractures. This figure is expected to increase by 50% by the year 2025 [1]. Of the 1.5 million new fractures annually attributed to osteoporosis, more than 20% involve complications in healing due to massive tissue loss or non-union defects [2]. Approximately 300,000 hip fractures occur annually, with a 24% average mortality rate in the first year. The survivors generally lose functional independence and require continued long-term care and ambulatory assistance [1]. The appeal of using biodegradable engineered tissue in corrective procedures is the promise of long-term success of the implant, a rapid post-operative recovery time, and an improved quality of life following the procedure [3-4]. It is in this way we look to improve on current technology.
1.2.1. Bone Grafts

The current gold standard for bone repair is the bone graft, possible when the amount of tissue needed is small [5]. In the most frequent cases involving an autologous graft, where the patient’s own tissue is harvested from a healthy site, the implant does not elicit an immunogenic response and is osteoinductive, and in that way is a successful therapeutic technique for bone repair. It does, however, suffer from serious shortcomings. Late graft fractures are common for these implants due to incomplete replacement of the tissue by host bone. It is reported that 60% of grafts fail within 10 years [6].

At the donor site, the procedure for harvesting the autologous bone requires invasive, painful surgery that can cause donor site morbidity (various diseased conditions), chronic pain, and infection in previously healthy tissue [7]. Of the estimated 1 million autologous bone grafts performed annually, approximately 12.5% of patients continue to experience pain in the donor site two years following the procedure [8]. In efforts to eliminate surgical trauma to the patient by harvesting autologous bone grafts, allogeneic and xenogeneic grafts have become accepted as alternatives. An allogenic graft is tissue harvested from a different member of the same species, and a xenogenic graft is tissue harvested from a different species than the patient. In these cases, the graft material is not immunogenically benign as in the case with autologous tissue. It must therefore be processed and irradiated to remove any immunogens. Unfortunately, as the necessary processing removes the bone morphogenic proteins which recruit mesenchymal stem cells to the implant site, it also negates the benefits of using bone material, drastically reducing the effectiveness of the treatment [5].

1.2.2. Metals and Ceramics in Bone Replacement

Due to the numerous shortcomings in natural bone grafts, the use of metals, such as alloys of titanium [9] and stainless steel [10], or ceramics, such as calcium phosphates [11], alumina [12],
and silica glass [13], has emerged as a way to permanently replace bone in a defect site. However, these replacement procedures come with critical drawbacks.

Metals are too stiff, with compressive moduli of up to hundreds of GPa, an order of magnitude greater than those of natural bone [14]. The highly stiff material causes stress shielding of other load-bearing bones and disrupts normal regulatory pathways induced by normal stresses, compromising the tissue surrounding the implant and leading to bone resorption [3]. Metals must also be surface-treated either with plasma, by ion impregnation, or other chemical means, in order to be biocompatible and allow growth of tissue and bone integration on the surface [15]. Ceramics have the proper porosity and modulus for the application, but in general are too brittle and can easily break or fracture [16].

The failure of both metals and ceramics to perform the non-structural functions served by native bone drastically hinders their long-term success. Aside from bearing compressive loads, natural bone acts as a source of mineral ions when blood levels of these minerals are low. Additionally bone is vascularized and allows for blood flow to act as a nutrient source, and routinely performs regulatory functions [4]. This shortcoming of non-organic implants has been shown to cause adjacent site necrosis, tissue recession from the implant, and subsequent implant loosening. The average lifespan of a bone replacement implant is 15 years and requires multiple corrective surgeries. Ironically, most implant recipients are elderly patients whose bodies cannot withstand the stress of repeated surgeries, and whose recovery times are inherently long and uncomfortable, while the younger recipients suffer from the short lifespan of the implant [17].

1.2.3. Polymers as an Alternative

Several polymers – both natural and synthetic – are of great interest in the field and are being investigated for use as tissue engineering scaffolds [18]. This class of materials is so diverse and
versatile that it can be made suitable for many applications. However, finding an ideal material has proven to be a challenge. One promising biopolymer is collagen, which is a key component of the extracellular matrix produced by differentiated osteoblasts during bone formation [19]. It has shown great potential for other tissue engineering applications, or in composites of other materials, but by itself lacks the compressive strength to be applied to bone regeneration [20]. Furthermore, at more than $150/g, collagen is quite costly and its use in a large implant would likely be prohibitively expensive for the average patient. Other popular natural scaffolds include fibrin and hyaluronic acid hydrogels [21], which possess similar limitations.

With the development of polymer science, some new synthetic polymers have emerged as potential candidates for tissue engineering. Some common choices are poly(ethylene glycol) (PEG) [22], poly(lactic acid) (PLA) [23], poly(L-lactide-co-glycolide) (PLGA) [24], and poly(ε-caprolactone) (ε-PCL) [25-26]. These, however, still fall short of the threshold values of mechanical properties [27]. They require the use of harmful organic solvents for polymerization and processing – solvents which must subsequently be removed completely from the polymer matrix and which make cellular and signaling molecule incorporation difficult [21]. Furthermore, they also tend to be custom polymers, which once again can become fiscally prohibitive.

If one were to overcome the challenges presented by these natural and synthetic polymers, many of them would still not be suitable implant materials without undergoing a surface modification procedure, be it to increase the roughness of the scaffold [28], present adhesion moieties on the surface [29], or change the chemistry of the surface to make the material more biocompatible [23]. These limitations create the need for an alternative. We believe that whey protein isolate, used as the base material, can bring us closer to designing a viable scaffold material free from the obstacles previously discussed.
1.2.4. Characterization Void for Whey Protein

As an important staple in the food industry, whey protein and its components have been subjected to in-depth characterization and study, though primarily as they relate to food science and engineering [30-31]. In various journals information can be found concerning the rheological properties of whey protein solutions of less than 10% WP [32], the onset of gelation of protein solutions below 20% [33], and extensive information on properties such as flavor, foaming, texture, film properties, and the like [34]. Much scarcer, however, is information on whey protein gels of high protein content, such as the range in the work proposed here. Only a select few publications discuss these gels, and those do not provide a thorough characterization of the material based on compositional and process variables. We seek to fill this gap in information by characterizing these gels of unique properties and learning to tune these properties towards the end function of the material.

1.3. Rationale

1.3.1. Therapeutic Scheme

The central dogma of tissue engineering is followed in this work. By the accepted mechanism, a solid, biodegradable, highly porous scaffold is constructed and seeded with signaling molecules and/or autologous cells harvested from a patient. After in vitro culturing, the scaffold is implanted into a bone defect site in the patient. Over a span of time, the scaffold begins undergoing resorption by the body while actively providing nutrients, structural support, and a geometrical template for the proliferating cells. The cells begin to mature and lay down an extracellular matrix, and finally mineralize the matrix as the final traces of the scaffold degrade, leaving behind intact, healthy bone. The degradation products are completely biocompatible and are broken down and removed from the body through normal physiological function [29, 35].
1.3.2. Material Selection

Bovine whey protein has been shown to promote the growth and differentiation of osteoblasts in different species [36-38] and to suppress osteoclast activity, preventing bone resorption [39]. Whey protein isolate is extremely inexpensive and abundantly available. Recent years have shown an increased drive to develop uses for whey protein in order to increase the value of milk products and reduce disposal costs and organic pollution [40-41]. Whey is considered a byproduct in cheese production, and the cheese manufacture industry pays for its disposal, as whey constitutes 80-90% of the original milk volume [42]. One novel application of whey protein inspired this project. The study investigated the use of whey protein gels as non-fouling filtration membranes – the properties of which are similar to those of the proposed naïve gel [43].

The components of the protein mixture are well characterized [31] both in structure and in sequence [44-45], and its gelling properties have been extensively studied and are favorable for the application. Its immunogenicity has been assessed in mice, and following processing similar to that proposed in this work, WPI films have been found to be immunogenically benign for up to 60 days [46]. The proposed study is based on the theory that by using whey protein isolate as the support and by changing the composition and processing methods, we can construct a strong gel that is fine-tuned to optimal scaffold characteristics.

Calcium chloride is added to improve gelling properties [47]. In an extensive study covering different salts and their relative impacts on the viscosity and gelation ability of whey protein solutions, calcium chloride ranked among the best gel-inducing salts [48]. These results have since been reproduced in other studies [33, 43], making the precursor suspension similar to the well-studied solutions of lower protein content. Furthermore, in efforts to construct a material that will perform an active role in bone regeneration, we believe that a calcium salt would be the
most suitable choice to incorporate into the gel, as it may serve as a calcium source for mineralization in late stages of tissue formation.

The contributions of various polysaccharides as additives and reinforcing agents are investigated in this work. Since natural biopolymers are generally mechanically unsuitable for load-bearing applications, the need for a composite material is likely to arise. Nanocomposites have been shown to drastically enhance the mechanical properties of a polymer matrix [49], and the same change in behavior is pursued here. Polysaccharides were selected due to the proven ability of cellulose to reinforce a polymer matrix [50-51], and because it is hypothesized that their hydrolytic degradation products may serve as an added nutrient source for proliferating cells. A built-in nutrient source would improve the growth and mineralization characteristics and expand the feasible scaffold dimensions – generally physically limited by insufficient diffusion into the scaffold interior [52]. Overcoming this limit would allow for use of the scaffold in the sites of major tissue loss that pose the greatest difficulty for existing therapeutic treatment methods.

1.4. Goals and Objectives

The overall goal of the project is to design and optimize a composite material that is biocompatible, biodegradable, and possesses material characteristics making it ideal for use as an implantable scaffold for bone regeneration. To that end, WPI gels were subjected to extensive characterization, and the information found therein was used towards the design of a feasible scaffold by pursuing several specific objectives:

1. To characterize the mechanical properties of WPI gels and WPI-based composites and optimize them for use as a bone tissue regeneration scaffold;

2. To determine the growth kinetics of preosteoblasts on the WPI gels and composites \textit{in vitro} and to characterize the effects of material and process variables on the cell growth;
3. To assess effects of material and process variables of WPI gels and composites on differentiation and mineralization of preosteoblasts cultured \textit{in vitro} on the gel surface;

4. To formulate the best combination of composite components and processing methods, for the best set of properties to be used in future studies.

In the chapters that follow, the material characterization and development process is comprehensively discussed and the product is evaluated for suitability for end use in bone tissue engineering.

1.5. References


Chapter 2: Assessing the Mechanical Properties and Biocompatibility of Whey Protein Isolate Gels Toward Use in Bone Regeneration

2.1. Introduction

Polymer scaffolds for tissue engineering have been under considerable study as a means of repairing tissue defects. The popular approach in many applications of engineered tissue is to introduce a 3D polymeric or ceramic scaffold seeded with cells into a defect site, where the scaffold provides structure, essential nutrient, and growth factors to the cells proliferating and differentiating in the defect site. While it provides a temporary template for the newly formed tissue, it is resorbed harmlessly by the body [1-4]. When the scaffold is intended for bone regeneration, achieving the proper mechanical characteristics, namely the strength and stiffness of the support, becomes integral to its success [5-6]. This study is meant to introduce whey protein as a promising base biopolymer in a scaffold for bone regeneration. We believe that by using whey protein as the support and by varying the composition and processing methods, we can construct a strong gel that can be fine-tuned for optimal scaffold characteristics.

Whey protein is a term used for a class of milk proteins (predominantly β-lactoglobulin, α-lactalbumin, and serum albumin) [7]. For several reasons, it is an attractive raw material that is suitable for the application. First, it is cheap and abundant. In recent years the drive has increased to develop uses for whey proteins in order to increase the value of milk products and reduce disposal costs and organic pollution [8-9]. The dairy industry wastes resources on the disposal of whey as a byproduct in cheese production, as whey constitutes 80-90% of the original milk volume [10]. Second, its constituent proteins have each been thoroughly characterized, primarily
for applications in the food industry [11]. Third, whey protein is heat sensitive so thermal denaturing can be done at low temperatures, making thermal curing of protein solutions straightforward. The structure of the material makes it a good choice for gel formation. Added calcium ions participate in cross-linking, hydrogen bonding, and hydrophobic interactions on cooling, thus tightening the network and forming a strong matrix [11]. Finally, the proven ability of bovine whey protein to promote the growth and differentiation of osteoblasts across species [12-14], while suppressing osteoclast activity [15] has led to this investigation of a novel application for whey protein. The role of osteoblasts is to construct and remodel bone tissue, while osteoclasts dissolve bone minerals and break down bone. Therefore in initial stages of bone regeneration, it is desirable to enhance the activity of the former while decreasing the activity of the latter.

The naïve material used in this study is a mixture of WPI powder (97.6% protein by weight), CaCl₂, and water. Calcium chloride was selected for its demonstrated ability to form a stronger whey protein gel than similar salts [16] and in order to incorporate calcium ions into the matrix to aid in cross linking and provide a calcium source for mineralization in future studies. The procedure used for gel formation was adapted from work in developing a separation membrane from whey protein [17]. The existing literature provides valuable information on whey protein solutions and gels at low concentrations – primarily as it relates to food science [18-20]. The purpose of this study is to enhance those results to the processability limit of the gel-forming protein suspension, with a focus on the end use as an implantable 3D scaffold.

The direct objectives of this work were threefold. The first was to characterize the tensile and compressive properties of high-concentration whey protein isolate (WPI) gels with respect to two variables: protein concentration and calcium chloride concentration, in order to reveal the
optimum composition for the desired mechanical properties. It is generally accepted that a compressive strength of 5 MPa and an elastic modulus of 50 MPa make a material suitable as a scaffold for bone regeneration [21-22]. The tensile properties were included to uncover any trend differences between the two testing directions and to provide a better understanding of the behavior of the material under stress. The second objective was to compare the morphological characteristics of the material at different compositions of the components and relate the differences to the macroscopic mechanical properties. The results were then used to select the compositional region of interest for future studies of the material towards the target application. Finally, the third objective was to assess the biocompatibility of the material, which is a direct indicator of its suitability for use as a tissue regeneration scaffold. The novelty of this work is primarily in the suggested application of WPI to the field of tissue engineering, and secondly in the range of WPI concentrations under characterization.

2.2. Materials and Methods

2.2.1. Materials

All water used in this work was >18 MΩ polished water from a Direct-Q 3 water purification system (Millipore, Billerica, MA). The materials used were WPI powder from Davisco Foods International (Eden Prairie, MN), where powder composition was: beta-lactoglobulin 68-75%, alpha-lactalbumin 19-25%, bovine serum albumin 2-3%, immunoglobulin 2-3%; calcium chloride dihydrate from Mallinckrodt Chemicals (Hazelwood, MO); and phosphate buffered saline solution (NaCl, KCl, Na₂HPO₄, KH₂PO₄ from Sigma-Aldrich, St. Louis, MO). MC3T3-E1 subclone 4 preosteoblast cells were purchased from ATCC (Manassas, VA), and αMEM, penicillin/streptomycin, fetal bovine serum, and Fungizone from Invitrogen (Carlsbad, CA).
2.2.2. Scaffold Fabrication

Samples for material testing were prepared by adding WPI powder to an aqueous CaCl$_2$ solution of half the volume and double the concentration desired for the final mixture. The final compositions ranged from 20 to 45% w/v WPI and 0 to 50 mM CaCl$_2$. To achieve these high WPI-containing slurries, protein powder was gradually added to the aqueous solution, with vortexing to mix between steps. Then the mixtures were adjusted to the final weight ratio by adding water to achieve the target concentration and vortexing once more. The volume of slurry was kept at 20% of that of the vessel, to allow for effective vortexing. The resulting viscous precursor slurries were then cast into the desired sample geometries.

Samples for compressive testing were loaded into custom PTFE molds manufactured in-house. The molds were constructed to generate cylinders of 7.62 cm (3 inches) in length, and 10 mm in diameter. Samples for tensile testing were loaded into aluminum molds to generate type-IV dogbones as specified in ASTM D638 [23]. Gelation was induced thermally by curing at 80°C for 60 minutes (cylinders) or 45 minutes (dogbones) for similar energy input to the different geometries. The time gap was established based on previous results indicating that increasing cure time above 45 minutes results in no change in mechanical properties (See Appendix A for cure-time study). Additional time was used for the cylinders to compensate for the heat conductivity of PTFE vs. aluminum and for the increased characteristic length of the sample (5 mm vs. 1.5 mm). The samples were then cooled at room temperature for 10 minutes and removed from the molds. The cylinders were cut to 10 mm lengths using a diamond-blade rotating saw for an aspect ratio of 1. The finished samples were allowed to swell in PBS for 2 hours prior to testing to ensure proper hydration and achieve their final dimensions.
2.2.3. Ultimate Mechanical Testing

Compressive and tensile testing was performed using an Instron universal testing system (model # 4411, Instron, Norwood, MA) at a cross-arm speed of 5 mm/min until failure. Only samples with breaks near the center of the sample were included in stress/strain calculations and analysis to eliminate break artifacts related to geometry. The load-deformation data were converted to stress-strain curves, and the failure point and initial slope of each were identified. The stress was calculated as load per initial cross-sectional area and strain was calculated as the change in length divided by the initial length. The sets of samples were designed to test the full range of WPI concentrations forming a solid gel (20 to 45% w/v) at a constant CaCl$_2$ concentration, and a wide range of salt concentrations (0 to 40 mM) for constant protein concentrations: 30, 35%, and 40% w/v WPI.

2.2.4. In Vitro Testing

Mouse preosteoblast MC3T3-E1 subclone 4 (SC-4) cells were cultured in $\alpha$ minimum essential medium ($\alpha$-MEM) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 $\mu$g/mL streptomycin, and 2.5 $\mu$g/mL Fungizone (amphotericin B). The cells were incubated at 37°C in high-humidity and 5% CO$_2$ atmosphere.

Samples of WPI gels were seeded with MC3T3-E1 mouse preosteoblasts and cultured in vitro in order to assess the capability of WPI gels to support cell growth. A precursor WPI solution of 45% w/v WPI and 10 mM CaCl$_2$ was cast into the wells of a non tissue culture treated 12-well polystyrene plate and cured for 20 minutes at 80°C. The plate was then cooled to room temperature and sterilized under UV overnight. In each well, 1 mL of $10^4$ cells/mL cell suspension (passage 4) were used for static seeding, and the cells were cultured for 53 hours at 37°C in 5% CO$_2$ atmosphere and 99% humidity. The samples were then prepared for scanning electron microscopy in order to visualize the growth surfaces.
2.2.5. Scanning Electron Microscopy

To correlate the topography of WPI gels with mechanical data, sample cross-sections comprising a range of WPI and CaCl₂ concentrations were viewed by scanning electron microscopy (SEM). Samples fabricated for SEM were rinsed with phosphate buffered saline (PBS) and fixed by a 2%-glutaraldehyde/1%-formaldehyde fixative solution and rinsed three times with 0.1 M cacodylate buffer. A postfix in a 0.1 M cacodylate buffer/0.004 M glycine solution followed. The fixed samples were dehydrated via submersion in a graded series of ethanol and then dried by critical point CO₂. The dried samples were then mounted to SEM stages using double-sided conducting adhesive and sputter-coated with gold for 2 minutes at 10 mA plasma discharge. The samples were imaged under vacuum at 5 kV.

Samples from in vitro studies were processed in a similar fashion with the following modifications. The surface of interest was already exposed, so the samples were not fractured, but were cored so-as to conform to sample-size specifications of the stage. Also, prior to ethanol dehydration, the samples underwent a post-fixation step with osmium tetroxide.

2.2.6. Statistical Analysis

Data are presented as means of at least three replications and standard deviation. Statistical significance was determined by performing a Studentized Tukey test (α = 0.05) for every paired means in the mechanical testing data.

2.3. Results and Discussion

2.3.1. Concentration Effects of Whey Protein Isolate

2.3.1.1. Mechanical Properties

The compressive and tensile properties of the whey protein gels were shown to be functions of WPI concentration (Figs. 2.1-2.2). The ultimate compressive strength increased with protein
concentration, up to a point, then reached a maximum and decreased (Fig. 2.1). The maximum strength observed was 1.79 ± 0.21 MPa, and corresponded to 35% w/v WPI at 10 mM CaCl$_2$. The tensile strength increased proportionally with protein concentration. The highest tensile strength observed was 0.47 ± 0.04 MPa, and corresponded to 45% w/v WPI at 10 mM CaCl$_2$. Several noteworthy features arose from these results.

The first was that the ultimate tensile material strength was consistently lower than the corresponding compressive strength. This phenomenon is not unusual, as this property depends on the specific mechanism of failure, which is different in tension and compression [24]. When a compressive load is applied, the force is displaced into the collapse of the prevalent macropores observed in Fig. 2.3a-c, delaying the full failure of the material. Under a tensile load, the pores cannot offset the force, and the cross-links between protein molecules dictate the failure – resulting in lower strength. The scarcity of pores in Fig. 2.3a supports this theory, as the difference in strength for this gel between the two testing directions is the smallest in the range.
The next noteworthy feature was the failure trend under compression, which occurred against expectations, though it proved repeatable and statistically significant. Numerous studies in the past determined the correlation between protein concentration and mechanical and rheological properties of whey protein isolate gels or the individual components of WPI. These studies used a variety of conditions, fabrication methods, and gel compositions, and a linear relationship was consistently found. However, these studies have categorically used protein concentrations markedly lower that the range in this study. Van den Berg et al. made their characterization using 3% and 5% WPI solutions [25]. Mulvihill and Kinsella, and Kuhn and Foegeding used pure β-Lg and WPI solutions, respectively [26-27]. The closest conditions to the ones in this study were used by Ju and Kilara, who tested heat-induced WPI gels of up to 18% WPI [28-29]. Since
protein concentration has such an impact on gel structure and properties, it is impossible to extrapolate these results to the range addressed in this work. The trend, then, with a peak strength reached between 35 and 40% w/v WPI, can be attributed to a number of phenomena.

A peak strength suggests competing effects. As the protein concentration increases, the solid fraction increases and replaces the void fraction in the gel, thereby increasing the material strength. However, at constant salt concentration, as protein concentration increases, the protein-to-salt ratio increases and above the critical protein-to-salt ratio, there are not enough calcium and chloride ions to effectively screen all charges on the protein molecules. Insufficient charge screening results in loose protein molecules unassociated with any aggregate, interfering with network formation and weakening the gel. In addition, the added void volume in the gels likely contributes to the phenomenon, since the SEMs show an increased porosity as WPI content increases. The decrease in material strength between 35% and 45% w/v WPI could also be explained by the change in the type of gel structure formed, as made evident by the images in Fig. 2.3 and discussed in detail in Section 2.3.1.2.

Both the compressive and tensile elastic moduli of the gels followed a linear relationship with respect to WPI concentration (Fig. 2.2). The compressive modulus increased linearly with protein concentration, with the highest observed being 2.37 ± 0.09 MPa. The modulus corresponding to the highest-strength sample (35% w/v WPI) was 1.65 ± 0.02 MPa. The highest observed tensile elastic modulus was 3.41 ± 0.08 MPa which corresponded to 45% w/v WPI and 10 mM CaCl₂. This relationship is well supported by the studies cited above for mechanical testing of WPI gels with lower concentrations, and was found to hold for WPI gels in this concentration range. The trend is caused in part by the crosslink density of the gel, which increases with protein concentration. It can be seen from Fig. 2.2 that the tensile elastic modulus
is higher than the compressive modulus throughout the range, though theory dictates that no systematic difference should exist between the two. The observed deviation stems from the compressibility of the gels, which contain both macro and micropores, which are collapsed by the compressive load and serve to dissipate it, while in tension, the pores cannot offset any portion of the load, and the actual material stiffness is obtained.

2.3.1.2. Gel Morphology and Microstructure

Scanning electron micrographs of gel cross-sections of different WPI concentrations at 10 mM CaCl$_2$ were used to correlate the mechanical behavior observed to the microarchitecture of the gels (Fig. 2.3). The effects of protein concentration on the macrostructure can be seen in Figs. 2.3a, b, and c, which represent gels of WPI concentrations of 20, 35, and 45% WPI, respectively. A distinct increase in both porosity and pore size is observed as protein concentration is increased. Additionally, at higher protein concentrations, greater pore interconnectivity can be observed (Figs. 2.3b, c). The differences observed were caused by the varying viscosities between the precursor slurries. The higher WPI slurries were more viscous and able to support larger air bubbles, which became pores upon curing. The thinner slurries were unable to entrain the air and the bubbles escaped into the ambient air. The scarcity of pores in Fig. 2.3a helps explain the difference between compressive and tensile ultimate strength, as the difference in strength for this gel between the two testing directions is the smallest in the tested range.

The microstructure of the same gels can be seen in Figs. 2.3d, e, and f. These images suggest a direct correlation between WPI concentration and the surface roughness of the cross section. In other words, the surface of the low-WPI gel is relatively smooth and flat (Fig. 2.3d), while the higher-WPI gels are rougher with a more globular structure (Figs. 2.3e, f). The images in Fig. 2.3 suggest a phase-separated microarchitecture of the gel, which transitions from a coarse-grained
structure – consisting primarily of a fine-stranded network, connected by fine strands of associated protein, to a biocontinuous structure – characterized by small aggregates interconnected by aggregated strands, to a protein-continuous structure – characterized by large protein aggregates mostly separated by the aqueous phase.

2.3.2. Concentration Effects of Calcium Chloride

2.3.2.1. Mechanical Properties

Two sets of gels were tested to failure in compression. One was at 30% w/v WPI, and the second at 35% w/v WPI – the concentration resulting in the highest observed mechanical strength. The effects of calcium chloride on the compressive strength of the material were evident but difficult to quantify, since gels made with a concentration greater than 20 mM CaCl$_2$ compressed uniformly to greater than 90% deformation without failure. The results for gels of lower concentration demonstrate an optimum strength, observed at 1.66 ± 0.34 MPa, corresponding to a concentration of 7.5 mM CaCl$_2$ and 35% WPI (Fig. 2.4). Under tension, two sets of gels were also tested – this time, one was at 30% w/v WPI (for comparison to compressive results), and the second at 40% w/v WPI – as a change less than 10% in WPI concentration exhibited no statistical difference in tensile properties. At both protein concentrations, the ultimate tensile strength showed the same trend with respect to CaCl$_2$ concentration as the compressive strength (Fig. 2.4 inlay). The maximum tensile strength achieved was 0.44 ± 0.05 MPa and occurred at a CaCl$_2$ concentration of 10 mM and 40% w/v WPI. As before, the compressive strength was consistently higher that the tensile strength.

The compressive modulus was determined to be a weak function of CaCl$_2$ concentration (Fig. 2.5), and is available for the full range tested, since it is a property inherent to the initial behavior of the gel under compression. A maximum compressive modulus of 1.96 ± 0.15 MPa was
obtained at a concentration of 7.5 mM CaCl\textsubscript{2} and 35% WPI. The tensile elastic modulus of the gels was shown to be a stronger function of CaCl\textsubscript{2} concentration at the WPI concentrations tested, particularly at 30% w/v WPI (Fig. 2.5). The maximum tensile elastic modulus achieved was 4.95 ± 0.22 MPa and corresponded to a CaCl\textsubscript{2} concentration of 0 mM and protein concentration of 40% w/v WPI. These findings are well-supported by results in other studies of heat-induced WPI gels. Mulvihill and Kinsella have found a peak strength and elastic modulus at 10 mM CaCl\textsubscript{2}, but did not test gels at 7.5 mM CaCl\textsubscript{2}, where the peak moduli of elasticity were found in this work [26]. The difference in the experiments lies in the use of pure β-Lg at 10% protein by Mulvihill and Kinsella, versus the use of whole WPI at concentrations between 20% and 45% in this study. As expressed previously [29-30], an important indicator of the hardness of WPI gels is the aggregate size in the precursor solution. It is this property that calcium chloride affects, leading to gels of
varying strengths for varying CaCl$_2$ concentrations. Added calcium chloride results in larger aggregates, and aggregate size prior to gelation inversely affects gel hardness [28]. Although this relationship was determined at lower WPI concentrations using material hardness vs. the failure stress used in this study, it is consistent with the trends found here and serves to support them (Figs. 2.4-2.5). Knowing that the trends in the literature hold for this range of WPI concentrations is essential to scaffold design and the use of composition towards the optimization of mechanical properties.

2.3.2.2. Gel Morphology and Microstructure

Scanning electron micrographs of gel cross-sections for varying CaCl$_2$ concentrations at 30% w/v WPI (Fig. 2.6) and 40% w/v WPI (Fig. 2.7) were used to correlate the mechanical behavior observed to the microarchitecture of the gels. The effects of CaCl$_2$ concentration on the gel macrostructure can be seen in Figures 2.6a, b, and c, as well as Figures 2.7a, b, and c, which represent gels of CaCl$_2$ concentrations of 0, 10, and 40 mM, respectively. Direct comparison of Figures 2.6a-c or Figures 2.7a-c suggest that although the increased calcium chloride in the gels may contribute to an increased frequency of macropores present in the gels, the pore size is independent of this variable. On the other hand, a pronounced increase in surface roughness can be attributed to the increased CaCl$_2$. This finding makes it possible to select the concentration of calcium chloride to optimize the mechanical properties of the gel without further consideration for the macropore structure, which is found to be dictated only by the viscosity of the precursor slurry, and pre-gelation air content.

The microstructure of the same gels is visible in Figures 2.6d-f and 2.7d-f. The structures in these images reveal the direct correlation between calcium chloride concentration in a gel and its roughness, nanoporosity, and structural globularity. These properties increase with the added salt.
These results are strongly supported by the findings in the work by Ju and Kilara [29], and Caussin et al. [30], which relate WPI gel microstructure to pre-gel size of protein aggregates. Also noteworthy is the ability to form a self-supporting gel in this study without the addition of salt. In some studies the addition of salt was essential to heat-induced gelation [26-27]. These studies generally used a maximum protein concentration of 10%. Others, including Caussin et al., have formed self-supporting gels in absence of salt, and found that a high WPI concentration is sufficient in forming the aggregates that result in gelation upon heating [30]. The aggregation behavior previously described is readily observed in comparison of Figs 2.6d, e, and f. With no calcium chloride (Fig. 2.6d), the gel is exceedingly smooth and the small aggregates are scarcely
discernable from the connections between them. On the other hand, with 10 mM CaCl₂ (Fig. 2.6e), the aggregates are larger and form a globular structure with a more prevalent nanopore network. This structure is even more pronounced in the 40 mM CaCl₂ gel (Fig 2.6f), where the mean aggregate size approaches the micrometer scale. Furthermore, for each calcium chloride concentration, Figs. 2.6 and 2.7 can be compared to reveal the additional aggregation behavior of the gels in Fig. 2.7, which contain a higher protein concentration.

It has been established that a rough scaffold surface improves its osteoconductive properties, or promotes the ingrowth of tissue into the scaffold [31]. By this standard, the gels of high WPI content and all but the lowest salt content exhibit the desired morphology for tissue regeneration.
2.3.3. In Vitro Scaffold Performance

SEM micrographs of the 2D WPI gel surface prove the ability of the material to support the adhesion and proliferation of MC3T3-E1 mouse preosteoblasts (Fig. 2.8). In addition to cell viability, the morphology of the cells can be assessed. The morphology was found to be flat and stellate, with pronounced filopodial extensions after 53 hours in static culture (For additional micrographs of cell growth on WPI see Appendix B). This is the desired phenotype expected for growth on a tissue scaffold. Flat, large cells indicate good adhesion and a high affinity to the substrate, and the presence of filopodia suggests active cell motility – essential to uniform population of a scaffold.

This finding reveals some distinct advantages of whey protein as a scaffold. In order to show favorable adhesion and proliferation characteristics, whey protein gels require no coating, physical or chemical surface modification, or bioactivation. WPI also requires no hazardous solvents during processing; rather, the neat fabrication is carried out entirely in the aqueous phase.

A potential pitfall for the use of any material for implantation is the possibility of an adverse immune response. In the case of WPI, which contains proteins of known immunogenicity in hypersensitive individuals, the concern is increased. However, this immune response is largely based on tertiary structure of the protein. While it is not mitigated by the harshly acidic
gastrointestinal conditions, it is greatly reduced by heat denaturation and at alkaline conditions as those used in this study [32]. Additionall, Rouabhia et al. demonstrated the suitability of WPI for implantation by conducting in vivo studies for up to 60-day finding WPI-based films to be non-immunogenic [33].

A few deviations exist between the properties required for a successful bone regeneration scaffold and those of the gel materials constructed for the study. First, in order for the gel to be used as a tissue regeneration scaffold, the porosity and interconnectivity must be significantly higher than in the gels used in this study. However, increasing the porosity by modifying the processing technique can be accomplished without difficulty, and can improve the suitability of the gel as a scaffold. Second, a clear limitation in this study is that with no additives to the naive material, both the break stress and the modulus of elasticity fall below the desired values for an implantable bone scaffold. However, this setback is not uncommon even among materials considered the frontrunners in the quest for the ideal bone scaffold material. Collagen and hyaluronic acid – both under extensive investigation for use in bone regeneration scaffolds, have been found wanting in terms of mechanical properties in pure form [34-35], and therefore are often used in composites which serve to correct this flaw. This strategy will be applied in this case. In fact, a recent study has shown that nanocomposites using nanocrystalline cellulose as the dispersed phase in a waterborne polyurethane matrix have resulted in a threefold increase in the tensile strength and an increase in the elastic modulus of up to three orders of magnitude [36]. A similar study achieved an enhancement in mechanical properties using simple and functionalized nanocrystalline cellulose as reinforcing fillers in a cellulose acetate butyrate matrix [37]. Based on those findings, it is likely that the drawback with naive WPI can be overcome by using WPI gel as the dispersing phase in a nanocomposite, where the material properties can be increased to
the appropriate range. Future work will investigate this class of nanocomposites, and the contribution of a dispersed phase to the mechanical properties, using the optimal operating range determined in this work – between 35% and 40% w/v protein and between 0 mM and 15 mM calcium chloride. Overcoming this hurdle would allow a material based on the gels examined here to become a viable option for use as a bone scaffold material.

2.4. Conclusions

The dependence of the mechanical properties of high-concentration whey protein isolate gels on protein concentration and concentration of calcium chloride was characterized in detail. The highest material strength can be achieved by using the optimum composition found here to be between 35% and 40% w/v WPI and 5-15 mM CaCl$_2$. The behavior of the elastic modulus follows the same trend as low-protein solutions and increases linearly with protein concentration. Charge screening plays an important role in determining the material strength and stiffness, and becomes more important at higher protein concentration. The ultimate strength and modulus necessary for a scaffold material for bone regeneration are likely achievable in a composite material based in the naïve gel examined in this study. The gels have demonstrated the ability to support the adhesion and proliferation of preosteoblast cells. The cells appear to have a high affinity to the material, as demonstrated by the proper phenotype that was observed.

2.5. References


Chapter 3: Measuring Hydration Characteristics of High-Concentration Whey Protein Isolate Hydrogels

3.1. Introduction

Hydrogels are hydrophilic, crosslinked gels that absorb water in large quantities without dissolving [1]. One of the most frequently addressed features of hydrogels is their swelling capability [2-3], caused primarily by osmotic pressure. The discrepancy of counterions between the inside of the gel and its surroundings – caused by ionic side groups – drives water into the gel [4-5]. Simple, macroscopic methods to characterize the hydration and swelling of the gel include measuring dimensions and mass of the gel as a function of time [6]. The values are compared to those at a reference state, often chosen as gelation conditions [7].

Hydrogels made from both natural and synthetic polymers are extensively used in tissue engineering research [8]. The ECM-like structure and swelling characteristics are desirable in their potential for carrying cell-signalling molecules and migrating parenchymal or progenitor cells into the construct. By swelling, the gel also expands its mesh and can be used to deliver previously trapped signaling molecules pre-loaded into the gel [9].

Gelation of WPI has long been studied, and its characteristics and parameters are extensively covered in the literature [10-14]. However, little information is available for WPI gels of concentrations greater than 20% WPI. They are generally not addressed in dairy or food science, as they do not originate from a fully-dissolved solution of WPI. Nonetheless, these gels have properties that can be applied in fields outside food science, and the characterization of their hydration and swelling properties may be of interest.
The goal of this study was to determine the hydration behavior of high-concentration WPI hydrogels. First the initial water content in the gel as a function of WPI was determined, then the hydration and swelling kinetics were characterized by weight and volume increase due to buffer uptake.

3.2. Materials and Methods

3.2.1. Materials

Experiments were conducted using WPI powder from Davisco Foods International (Eden Prairie, MN), calcium chloride dihydrate from Mallinckrodt Chemicals (Hazelwood, MO), and polished water (>18 MΩ) from a Direct-Q 3 water purification system (Millipore, Billerica, MA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

3.2.2. Hydrogel Fabrication

The WPI gels were fabricated as previously described [15]. Briefly, WPI powder was mixed into aqueous CaCl₂ solutions in varying concentrations, and vortexed to mix. Water was added to adjust to weight, and reagitated to mix. These gel precursor suspensions were cast into PTFE molds and gelled thermally at 80°C for 60 minutes. The cylindrical gels were cooled for 10 minutes before removal from the molds, and cut into lengths for repeated samples.

3.2.3. Water Uptake Measurements

The freshly-gelled samples were weighed, measured for diameter and length using microcalipers, and submerged in 0.1 M phosphate buffered saline (PBS) for 24 hours. At various times during this interval, the gels were each removed, lightly wiped with an absorbent wipe to remove excess moisture, measured for weight and dimensions, and resubmerged in PBS. A second set of gels was initially weighed and measured as before, but instead of being hydrated, was allowed to air-dry until no further mass loss was observed.
3.2.4. Power Law Fits

The mass and volume swelling data for each composition were normalized to the initial mass or volume of the sample and expressed as percent increase over the initial values. The normalized data for each composition were fit to a power law model (Eq. 3.1) using SAS statistical software. The regressions were performed using the Levenberg-Marquardt minimization algorithm, with 1 and 0.01 as initial guesses for parameters $a$ and $b$, respectively. Parameter $b$ was used to compare the swelling properties of the different gels.

$$Q = a \cdot t^b \quad \text{(Eq. 3.1)}$$

3.2.5. Statistical Analysis

The data are presented as means of three replications. The convergence criterion for the objective function was set to $1 \times 10^{-6}$ in all regressions. The Wald test in SAS was used to calculate 95% confidence intervals corresponding to the regression parameters.

3.3. Results and Discussion

3.3.1. Initial Water Content

Freshly prepared gels of varying WPI concentrations were air-dried until their mass and dimensions remained constant. Initial and final mass and dimensions were recorded, and the difference was accounted for by the evaporation of water. The overall mass loss and volume loss are shown as functions of WPI concentration in Fig. 3.1a and 3.1b, respectively. Not surprisingly, the higher the WPI content of the gel, the less water was lost, because less water was initially present in the matrix. The trends could be fit to linear models, with the intercepts forced to 1, for 100% mass or volume loss for the case where no WPI is initially present. The slopes were -1.2% and -1.3% for mass and volume, respectively. These slopes are in good agreement with a mass balance on the gel. For every 1% w/v WPI added to the gel, 1.2% decreased mass loss due to
drying was observed. The difference could be attributed to the slight variation in density between the WPI suspension and pure water, made significant by the volume-basis of the concentration. The volume change due to drying was greater than that of the mass. The shrinking is brought on primarily by interfacial forces acting on the inner surface of the gel [16]. Also, in absence of water, the protein chains enter a glassy state, becoming more compact to neutralize secondary charges [17].

3.3.2. Swelling and Power Law Fits

The mass and volume data were fit to power law models to describe the swelling kinetics of each gel. The regressions were performed for the purpose of comparing exponent b (Eq. 3.1) for the different gels and using the information to quantitatively assess the effect that concentration had on the swelling behavior of the gels. The power law models were effective in describing the shape of the swelling curves. Fig. 3.2 contains parameter values for the exponent as a result of the mass and volume regressions (See Appendix C for individual swelling curves, including standard deviation, and full SAS output files for non-linear regression). The mass-defined
swelling parameter was found to be a function of WPI concentration, but the volume-defined parameter remained independent of WPI (Fig 3.2a). CaCl$_2$ strongly affected the swelling parameter for both mass and volume of the gels. The value of the swelling parameter increased with CaCl$_2$ concentration, with the largest change between 10 mM and 20 mM CaCl$_2$. For concentrations between 20 mM and 50 mM, the swelling parameters remained relatively constant (Fig. 3.2b). For every tested composition, the mass-defined swelling parameter was higher than the volume-defined swelling parameter.

Theoretical models dictate that the swelling follow a power law with time, and that depending on the relative rates of diffusion and chain relaxation, the exponent should be between 0.5 and 1 [3]. The exponents found in this study were significantly lower, and the disparity can be attributed to the hydrating solvent. The diffusing solvent was 0.1 M buffer, so the driving force was weaker than would be expected for pure water – the solvent used for exponent prediction [17]. Although it detracted from the breadth of information attainable in these studies, the buffer was used because its ionic strength is more relevant to the swelling of the gel in a biological environment than that of water.
Mass change was better predicted by the power law fit than volume change for all samples across the compositional range, as determined by $R^2$ values. Furthermore, mass gain was higher than volume gain for every tested composition. The difference was greater for high WPI concentrations than low concentrations. This is likely caused by the macroporosity known to be present in high-WPI gels [15]. The mass increase is brought on by a combination of the filling of macropores and the diffusion of water into nanopores and between protein chains. The saturation
of macropores with water or buffer does not effect a pronounced change to the volume of the overall gel. The volume increase occurs as a result of water diffusion and the relaxation of the peptide backbones brought on by charge neutralization.

Additional factors affecting overall swelling of hydrogels are crosslink density, charge density of side groups, and the ionic strength of the solvent. The observed swelling trend with respect to WPI concentration could be explained by several factors. At low WPI concentration, macroporosity is limited and does not greatly inflate the mass change compared to the volume change, so a wider gap is seen in the mass change for gels of 20%-30% w/v WPI vs. 35%-45% w/v WPI, than is seen for the volume change (Fig 3.3a, b). Overall, the degree of swelling increased with WPI concentration as a result of the increased charge density created by increasing the protein content per volume of gel. The higher charge density raises the driving force controlling the flow of water into the gel, thereby increasing the rate and degree of swelling.

The crosslink density decreases the swelling by restricting the motion of protein chains. The crosslink density of the WPI gels is dictated by both covalent bonds between cysteine residues through disulfide bridges, and by secondary bonds by ionic and dipole forces [11, 13]. The covalent bond density scales with protein concentration, and thus acts as a competing force controlling the degree of swelling. At a certain threshold protein concentration, the crosslink density is high enough to mitigate the driving force for solvent infiltration, and at 45% w/v WPI, the gel exhibits less overall swelling than gels of lower concentration.

The observed dependence of swelling on CaCl$_2$ concentration is a result of three principal factors. First, the presence of added ions in the matrix of the gel increases the driving force bringing solvent into the gel [18]. Second, CaCl$_2$ has been shown to affect the nanoporosity of the material. As CaCl$_2$ content is raised, gel nanoporosity is markedly increased [15]. The added
nano-scale porosity is still many orders of magnitude larger than the size of the diffusing solvent molecules, so the pores behave much like the macropores described earlier. When the nanopores absorb water, the gel mass increases more than the gel volume, as is indeed observed in the increased discrepancy with increased CaCl₂ between mass-defined and volume-defined swelling (Fig. 3.4 a, b). Third, added ions from CaCl₂ participate in charge screening of WPI side groups.

Fig. 3.4 – Gel swelling curves at varying CaCl₂ concentrations. Water uptake in terms of (a) increased mass and (b) increased volume of gels with respect to time. Expanded views of short times for (a) and (b) are presented in (c) and (d), respectively. Data are represented as percent increase over initial mass or volume; curves represent power-law fits to the data.
creating larger and more tightly-bound aggregates [19]. This phenomenon is dictated by the WPI-to-CaCl\textsubscript{2} ratio. Thus, as CaCl\textsubscript{2} is added to the matrix, the charges of calcium and chloride ions, and WPI side groups, neutralize one another, mitigating the charge gradient driving the solvent flow into the gel. This neutralization behavior likely accounts for the observed saturation in swelling parameter reached at 20 mM CaCl\textsubscript{2}. Following 24 hours in buffer, the highest degree of swelling observed for a 10 mM gel was 24.1\%, corresponding to the mass of 40\% w/v WPI (Fig. 3.3a). The highest observed volume change was 10.5\%, corresponding to 45\% w/v WPI (Fig. 3.3b). The highest degree of swelling by mass observed for a 35\% w/v WPI gel was 33.2\% by mass (Fig. 3.4a), or 14.8\% by volume (Fig. 3.4b), both corresponding to 50 mM CaCl\textsubscript{2}.

The swelling kinetics are relatively slow compared to some gels under study [6, 20-21], but no actuation or sensing capability is required in the proposed system, and the application does not call for a fast-response hydrogel. Furthermore, the degrees of swelling observed for the various gels appeared low within the range for common hydrogels. However, the degrees of swelling were calculated using the gelation conditions as the reference state. When the full hydration range is considered – from dry, glassy gel to fully-hydrated gel – most gels reach approximately 85\% swelling in 0.1 M PBS. The gelation conditions are frequently chosen as the reference state [7], and are the appropriate choice for this application. Here, the WPI gel is formed by casting into a mold in the desired shape of the gel sample. For the sake of reproducibility and predictability of dimensions and properties, a 2-15\% change in sample dimensions is preferable to a 70-80\% change.

3.4. Conclusions

Complete drying of the gels showed that initial water content was linearly dependent on WPI concentration and agreed well with values which would be obtained by performing a mass
balance on each gel. The swelling parameters of WPI gels were characterized as a function of composition. Swelling was found to increase with increased WPI or increased CaCl$_2$ concentration. Water uptake during gel hydration caused swelling of up to 33.2% by mass and 14.8% by volume over the gelation reference state. The mass and volume changes due to hydration could be modeled by a power law, and the exponents resulting from the fit gave insight into the contributions of the gel components to the swelling behavior. Factors controlling the swelling were determined to be the gradient in charge density between the solvent and the gel, the crosslink density, and macro- and nanoporosity of the gels. All these factors were dictated in part by the composition of the gel and led to the observed responses.

### 3.5. References


Chapter 4: Dispersed Polysaccharide Composites as a Means of Enhancing the Mechanical Properties of Whey Protein Isolate Gels

4.1. Introduction

As a cure for major bone loss, bone tissue engineering is aimed at supplying a temporary therapeutic implant that allows and promotes the growth of native bone and complete removal of the implant from the site. The implant is a scaffold that temporarily serves to replace the missing tissue in its load-bearing capacity and nutrient source for surrounding tissue. During this time, the scaffold either contains or recruits osteoprogenitor cells into the site and provides a growth surface upon which the cells proliferate and differentiate into mature bone cells. The differentiated bone cells begin to deposit a proteinous extracellular matrix and to mineralize that matrix with calcium phosphate. This mineralized matrix comprises the immature bone, which then gets repeatedly remodeled for strength and structure, and establishes a blood supply, leading to mature bone in the site of treatment. During the growth and mineralization process, the scaffold is meant to gradually degrade into non-cytotoxic products that are harmlessly cleared from the body [1-5].

To fulfill this role, a bone regeneration scaffold must conform to a specific set of properties. It must contain an interconnected network of pores large enough to allow cell migration and adequate diffusion to enable internal cells to remain viable  [6-9]. It must be biocompatible, biodegradable, ideally promote recruitment of stem cells or osteoprogenitors, and stimulate the proliferation and differentiation of osteoblasts [10-11]. Also, the scaffold must possess compressive strength and stiffness sufficient for load-bearing capability while not being so stiff
that it shields the surrounding tissue from stresses. This is particularly important, since mechanical stresses are long known to be necessary for healthy remodeling of bone [12].

Whey protein isolate (WPI) hydrogel has shown considerable promise as a material for bone tissue engineering. WPI shows favorable gelation properties and processing ability in an aqueous environment. It has been shown to be non-cytotoxic in vitro and a suitable growth surface for osteoblast-like cells in long-term culture. These cells adhere to the non-modified surface with high efficiency, exhibiting the desired morphology and phenotype, and successfully differentiate and form bone deposits [13]. In vivo testing of WPI films showed WPI to be biodegradable and immunogenically inert, thus suitable for implantation [14]. WPI is also a highly inexpensive, abundant, and environmentally-sound material. Its principal drawback is that like many other natural and synthetic polymers under investigation for the application, its mechanical properties simply fall short of those required for appropriate mechanical loading of an implanted scaffold [15].

As mechanical properties remain a ubiquitous challenge for the use of polymeric, degradable scaffolds, composites using these polymer materials have grown in recognition as a means to mitigate the problem. Two-phase composites of various types and compositions have shown improved mechanical behavior compared to their pure-polymer counterparts. Ceramics [16], glassy blocks in block copolymers [17], polysaccharides [18-19], and carbon nanowires and nanotubes [20] have all been used as dispersed-phase materials in such composites to enhance the mechanical properties of a biologically appropriate scaffold material.

Polysaccharides are attractive as a dispersed phase for a polymer composite for use in bone regeneration. Cellulose, in particular, has been shown to increase the modulus of a polymer by
orders of magnitude in relatively small concentrations [18-19]. With that enhancement, the only hurdle observed for the use of WPI gel as a bone scaffold material is overcome. Provided it fulfills this purpose, a polysaccharide phase would be degradable by the body into shorter, biocompatible chains, and the right chemistry could provide an added nutrient source for proliferating cells [13].

The direct objectives of this work were to characterize the compressive properties of high-concentration whey protein isolate (WPI) gels with various polysaccharide additives with respect to two variables: polysaccharide type and concentration. It is generally accepted that a compressive strength of 5 MPa and an elastic modulus of 50 MPa make a material suitable as a scaffold for bone regeneration [21-22], and previous findings have shown that naïve WPI gels are approximately one order of magnitude lower in these properties. Using the results generated in this study, the compositional region of interest for the material could be identified towards success in the target application.

4.2. Materials and Methods

4.2.1. Materials

All water used in this work was >18 MΩ polished water from a Direct-Q 3 water purification system (Millipore, Billerica, MA). The materials used were WPI powder from Davisco Foods International (Eden Prairie, MN); calcium chloride dihydrate from Mallinckrodt Chemicals (Hazelwood, MO); chitosan from Polysciences Inc. (Warrington, PA); cellulose, amylopectin from maize, amylose, dextran, and phosphate buffered saline solution components (NaCl, KCl, Na₂HPO₄, KH₂PO₄) from Sigma-Aldrich (St. Louis, MO). All materials were used as provided without further purification.
4.2.2. Scaffold Fabrication

Samples for material testing were prepared by stepwise addition of WPI powder and polysaccharide powder (amylose, amylopectin, cellulose, chitosan, or dextran) to an aqueous CaCl$_2$ solution of half the volume and double the concentration desired for the final mixture. The final compositions ranged from 20 to 45% w/v WPI, 0 to 0.25 g polysaccharide additive per g WPI, with 10 mM CaCl$_2$. The mixtures were homogenized by vortex followed by a submerged wand mixer, and adjusted to the final weight ratio by adding water to achieve the target concentration. The precursor suspension was then cast into the desired sample geometry.

The samples were loaded into custom PTFE molds manufactured in-house. The molds were constructed to generate cylinders of 7.62 cm (3 inches) in length, and 10 mm in diameter. Gelation was induced thermally by curing at 80°C for 60 minutes. The samples were then cooled at room temperature for 10 minutes and removed from the molds. The cylinders were cut to 10 mm lengths using a diamond-blade rotating saw for an aspect ratio of 1. The finished samples were stored in PBS for 2 hours prior to testing to ensure proper hydration.

4.2.3. Ultimate Mechanical Testing

Compressive testing was performed using an Instron universal testing system (model 4411, Instron, Norwood, MA) at a cross-arm speed of 5 mm/min until failure. The mechanism for failure was the buckling of the gel in failure lines parallel to the direction of applied force. The load-deformation data were converted to stress-strain curves, and the failure point and initial slope of each were identified. The sets of samples were designed to test the full range of each polysaccharide composition to the processing-ability limit of the casting suspension, and subsequently, a full range of WPI concentrations forming a solid gel (20 to 45% w/v) at a constant amylopectin concentration was tested.
4.2.4. Statistical Analysis

The data are each presented as a mean ± standard deviation of at least three replications per data point. Statistical significance was determined by performing a Studentized Tukey test (α = 0.05) for every pair of means in the mechanical testing data, or the Welch’s t-test for comparison of amylopectin composites to naïve WPI gels.

4.3. Results

4.3.1. Additive Type Effect

The different polysaccharide additives (Fig. 4.1) had varying effects on the composite material, but the predominant outcome of polysaccharide incorporation was the reduction in the strength and stiffness of the material. The compressive strength values, compressive moduli, and break strain values for the various scaffolds in this study are presented in Figs. 4.2, 4.3, and 4.4 respectively. All sets of composites were compared to a nominal, additive-free gel. The nominal value was taken as the average between all additive-free gels. Amylopectin was the exception to the general result, exhibiting an increased compressive strength and some evidence of increased modulus at a constant WPI concentration. In contrast, the tested amylose, cellulose, chitosan and dextran additives all caused a decrease in compressive strength, and all but chitosan caused a decrease in compressive modulus. The data suggest that low chitosan concentrations slightly improve the compressive modulus.

Fig. 4.1 – Molecular structures of polysaccharide additives. (a) Amylose, or amylopectin where amylopectin also containes α(1-6) branches; (b) cellulose; (c) chitosan; (d) dextran.
While the threshold values of compressive strength and modulus suitable for a bone regeneration scaffold are well defined, there is no agreed-upon value of deformation a scaffold should withstand before failure. The additives effected changes to the break strain of the material, but no assertion is made as to the impact on the quality of the scaffold due to this change. Amylopectin increased the strain at which failure occurred, and amylose tended to increase the strain. Cellulose showed little effect, and chitosan and dextran tended to make the material fail at lower deformation.

4.3.2. Additive Ratio Effect

The mechanical properties of each polysaccharide additive displayed a different concentration dependence. Any claim as to a trend exhibited by a set of composites is based upon 95% significance level as determined using the Tukey test. Amylose-containing composites showed a maximum strength at a ratio between 0.05 and 0.10 amylose/WPI, whereas higher ratios decreased strength below the nominal value (Fig. 4.2). The compressive modulus decreased
proportionally with amylose/WPI ratio (Fig. 4.3). The strain at which failure occurred qualitatively followed the trend seen for the material strength, but all strain values exceeded the nominal value (Fig. 4.4).

Amylopectin-containing composites with 35% w/v WPI followed a gradual sigmoidal increase in mechanical strength with increased amylopectin/WPI ratio (Fig. 4.2). The modulus did not exhibit a consistent pattern, but no composite had a modulus significantly higher than the nominal, while for several composites the moduli were significantly lower (Fig. 4.3) – a result that was later found to be anomalous in a range of protein concentrations. For amylopectin, as for amylose, the break strain was qualitatively similar in behavior to the mechanical strength. A gradual sigmoidal increase was observed and supported statistically (Fig. 4.4). The two starch components have an identical backbone, but very different contributions to the composite (Fig. 4.5), suggesting that the difference between them – in the highly branched structure of amylopectin – is responsible for the change.

Cellulose-containing composites effected no consistent change to the properties tested. Variations were significant, but occurred in both directions for strength (Fig. 4.2). The modulus also did not exhibit a predictable trend with respect to cellulose/WPI ratio, but no ratio was significantly higher than nominal, while several were significantly lower (Fig. 4.3). The break strain remained nearly independent of cellulose/WPI ratio save an inconsistent experimental value for 0.1 g cellulose/g WPI (Fig. 4.4).
Chitosan-containing composites decreased in compressive strength proportionally with chitosan/WPI ratio (Fig. 4.2). The modulus did not exhibit a predictable trend with respect to chitosan/WPI ratio but suggests an intermediate maximum stiffness corresponding to approximately 0.10 g chitosan/g WPI (Fig. 4.3). Like the mechanical strength, the break strain decreased roughly proportionally with chitosan/WPI ratio (Fig. 4.4).

Compressive strength decreases first rapidly, then gradually to a plateau as dextran/WPI ratio is increased (Fig. 4.2). The compressive modulus follows the same relationship (Fig. 4.3). The break strain relationship is similar if less distinctly clear than the other two properties (Fig. 4.4).

4.3.3. WPI Concentration Effect

As the most promising of the tested polysaccharides, amylopectin was incorporated into gels of a range of protein concentrations to determine if its effect would carry across to other concentrations. Between the two components, WPI is the one that causes a greater effect upon – and thus dominates – the mechanical properties. The compressive strength and modulus of gels with constant amylopectin concentration and varying WPI content are plotted and compared to

Fig. 4.5 – Compressive strength (a) and compressive modulus (b) of amylose- or amylopectin-based composites. Composites contain 35% w/v WPI and 10 mM CaCl₂.
naïve WPI gels of the same concentration in Fig. 4.6. Amylopectin was found to bring about a significant enhancement of the mechanical properties of the gel. Surprisingly, it was found that the original results at a range of amylopectin concentrations was the least meaningful change in the range of protein concentrations. An increase of as much as 100% was observed for 40% w/v WPI compressive strength, and up to 60% greater compressive modulus was observed for the same gel. These results validate the initial observation of improved mechanical properties with an amylopectin additive.

4.4. Discussion

One of the most difficult obstacles for creating composites conforming to the desired property profile is poor interaction between the phases in the composite. If interphase attractive forces are too weak, or repelling forces occur, these forces undermine the mechanical integrity of the solid. As a consequence, the mechanical properties fall short of requirements and degradation and loosening of the particulate can take place [23-25]. It is likely that this phenomenon contributed

Fig. 4.6 – Compressive strength (a) and modulus (b) of amylopectin-based composite compared to naïve WPI gels. Composites contain 10 mM CaCl$_2$ and 0.77 g amylopectin per g WPI – corresponding to 0.2 g amylopectin per g WPI for 35% w/v WPI composite. Symbols correspond to difference between composite and corresponding naïve gel of significance of $p < 0.05$ (*), $p < 0.01$ (†), $p < 0.005$ (**), $p < 0.001$ (††), or $p < 0.0005$ (‡).
in part to the observations in this study. When polysaccharides were incorporated into the gel precursor suspension, they most likely formed secondary bonds with amino acid side groups. Since most of the side groups available for interaction in the polysaccharides used are hydroxyl groups, they’re likely to form weaker bonds than what is possible with protein-protein interactions, thus on the macroscopic level weakening the overall solid and reducing the stiffness.

Chitosan addition showed some increase in the modulus of the material, if a decrease in mechanical strength. The cause for the behavior may stem from chitosan’s backbone chemistry (Fig. 4.1c). A chitosan molecule contains many primary amines that become available for protein-polysaccharide interactions during and following gelation. This interaction would serve to make the gel more rigid.

Amylose and amylopectin have identical backbones, yet their contributions to the mechanical properties of the matrix in which they were dispersed were quite different (Fig. 4.5). The only differences in the molecular structure of the two is the high degree of branching by α(1-6) linkages in amylopectin which is absent in amylose [26] (Fig. 4.1a). The branching of the polymer, along with molecular weight, affects its water solubility, rendering amylopectin insoluble, which could contribute to the difference in effects. However, not cellulose, chitosan, or dextran is soluble in water and none of these demonstrates the behavior of amylopectin in the matrix, so the solubility is not believed to cause the change. The branching also causes molecular crystallinity in amylopectin as compared to amylose, which is generally helical until associated [27]. Successful additives for strength and stiffness in other studies have been crystalline in nature [18]. The crystalline structure adds to the stiffness of the dispersed phase and facilitates load transfer from the dispersing phase [25].
The applicable length scale of the additive has a great impact on the composite properties. By using the materials tested in this study in nanoparticulate form, the measured mechanical properties would be improved upon. Nanocomposites are known to display traits different from their conventional counterparts with a very small fraction of additive. The driving force for the change is the increased interfacial area between phases. By replacing microparticles with nanoparticles of the same material, specific surface area increases by three orders of magnitude. If the interaction between the dispersed phase and the matrix can be made favorable, the increased interaction can lead to radical enhancement of mechanical properties that cannot be achieved with conventional composites [28]. Thus, both amylopectin and chitosan have great potential for launching the mechanical properties of the gel matrix into the range suitable for bone regeneration. Also, because property enhancement hinges on interfacial interactions, surface-functionalization of cellulose with compatible surface groups – in this case amine or carboxyl groups – should encourage particle-protein interactions and would likely achieve similar results [19].

Considering the improvements in structure observed for the range of WPI concentrations with addition of amylopectin, the lack of change in mechanical behavior for the naïve optimal concentration, 35% w/v WPI, was peculiar. The naïve gel optimal behavior was previously attributed to the aggregate size and the size of interconnects or bridging between the aggregates [29-30]. Provided this is accurate, the incorporation of the additive may have interfered with the already optimum size and interaction of the aggregates during gelation, such that any improvement offered by the additive was nullified by the competing interference. Thus perhaps where there was room for improvement in the gel microstructure, as in the other concentrations, significant improvement was observed.
The amylopectin composite properties were better suited for application in bone tissue regeneration requiring load-bearing capability. However, in order for amylopectin to be considered as a viable additive to the naïve WPI matrix, it must be determined that the material does not interfere with cell adhesion and proliferation known to occur on the naïve WPI gel surface and vital to the function of the scaffold. Amylopectin has been previously used in drug encapsulation and delivery and is acceptable for use in the body [31-33]. It is not expected to disrupt cell seeding and growth on WPI surfaces, but the interaction will require testing in future studies.

The best composite tested in this study had a compressive strength of 3.50 ± 0.35 MPa and a modulus of 2.84 ± 0.17 MPa. Although showing significant and meaningful improvement over the naïve WPI scaffolds, these properties still fall short of the threshold mechanical properties dictated for their application. However, the mechanical strength has been raised to within 30% of the target strength. With changes to the dispersed phase in the form of mean particle size and functionalization, the necessary properties may be achieved.

4.5. Conclusions

Composite scaffolds were fabricated using varying compositions of whey protein isolate and different polysaccharides. Amylose, amylopectin, cellulose, chitosan, and dextran were evaluated as a dispersed phase in the WPI gel matrix in conventional, particulate form. In this form, chitosan improved gel compressive modulus over the naïve gel, but detracted from the compressive strength. Amylose, cellulose, and dextran all formed composites of inferior mechanical properties to those of naïve WPI gel.

Scaffolds containing amylopectin had higher compressive strength and modulus than naïve WPI gel, with up to a 100% improvement in compressive strength and up to 60% improvement in
modulus with 0.20 g amylopectin per g WPI. Amylose and amylopectin possess the same backbone, and since amylose incorporation detracted from the mechanical properties, it was determined that the backbone chemistry was not responsible for the enhancement seen in the amylopectin composites. The only difference between the two polysaccharides is the branching in amylopectin. It is therefore likely that the branching caused the difference in mechanical properties. Future experiments will determine what improvement can be gained by using nanocrystalline polysaccharides for fillers in nanocomposites of the material.

4.6. References


Chapter 5: Proliferation Kinetics and Mineralization Properties of MC3T3-E1 Cells on Whey Protein Isolate Scaffolds for Bone Tissue Regeneration

5.1. Introduction

Since inception, the field of tissue engineering and regenerative medicine has gained momentum and recognition and is considered the long-term solution for large-scale tissue loss. In the specific case of bone tissue, the method involves the use of a temporary scaffold with the geometry and microstructure mimicking that of bone [1-2]. This scaffold fulfills several roles. It acts as a temporary place-holder for the tissue that will grow in its place, serving the structural and, where applicable, load-bearing functions of native bone. It acts as a geometrical template, or blueprint, for the growing tissue [3]. Concurrently, osteoinductive scaffolds are able to recruit cells into the bone-deficient site from surrounding tissue and promote proliferation to achieve a fully populated scaffold while also inducing differentiation into osteoblasts. Osteoblasts begin to lay down the extracellular matrix and mineral deposits that comprise immature bone tissue [4-5]. The bone tissue continues to be formed and remodeled on a time scale comparable to that of the degradation rate of the scaffold. The result of this process is that the site of bone loss is restored by the body itself with native bone, and the scaffold – having fulfilled its purpose – is eliminated from the site [6]. The need for a proposed scaffold to allow for and even promote the adhesion and differentiation of osteoprogenitor cells is evidenced by this mechanism of therapy. Previous work has shown promising characteristics for bone tissue regeneration using whey protein isolate (WPI) gels [7]. WPI is an attractive material since it is highly abundant, natural, and inexpensive. It is a byproduct in cheese manufacture and is rarely used outside the food industry. It was found
that the mechanical properties are on scale with many other materials under investigation for the bone tissue regeneration application, and that preosteoblast cells introduced to the system readily adhere to the surface and form a viable culture and display a healthy morphology. In addition, WPI is fully biocompatible and biodegradable, and has demonstrated certain immunomodulatory properties which may aid in combating infection upon implantation [8]. When processed under the conditions studied here, it has been shown not to effect an immunogenic response in vivo [9], as WPI immunogenicity stems largely from tertiary structure that is destroyed in these conditions [10]. It is also readily processed in a fully aqueous environment – eliminating the need for hazardous solvents – and its gelation can be induced thermally at low temperatures [11].

A major hurdle facing scaffold development is cellular maturation. Once cells fully mature into osteocytes, they cease to proliferate and serve no further role in bone formation [3]. Therefore, the rapid proliferation of cells populating a scaffold prior to differentiation is desirable, whether it takes place in vitro, in vivo, or ex vivo, so that upon differentiation the scaffold is uniformly and densely populated to give rise to strong, dense bone tissue. To that end, we conducted a study to quantify the ability of WPI gels to fulfill this role and the effect that composition has on scaffold performance. To achieve this goal, several specific objectives were pursued: 1) Assess the adhesion efficiency and proliferation rates quantitatively by comparing kinetic parameters to determine the effects of the scaffold composition; 2) Optimize scaffold composition with respect to non-differentiated cellular behavior; 3) Determine whether cells grown on the material are able to differentiate and produce a mineralized extracellular matrix. For accurate comparisons and reliable analysis, these studies were conducted in two-dimensional cultures.
5.2. Materials and Methods

5.2.1. Materials

The water used in this study was polished water, with resistivity greater than 18 MΩ, from a Direct-Q 3 water purification system (Millipore, Billerica, MA). Whey protein isolate was obtained from Davisco Foods International (Eden Prairie, MN) and used as provided. Unless otherwise stated, all other chemicals used were obtained from Sigma-Aldrich Corporation (St. Louis, MO).

5.2.2. Scaffold Fabrication and Processing

The scaffolds consisted of thermally-induced WPI gels of varying compositions. Whey protein was mixed into aqueous solutions of calcium chloride by vortexing to form suspensions ranging from 20 to 45% w/v WPI, from 0 to 20 mM CaCl₂, and from 0 to 0.25 g/g WPI of amylopectin. The suspensions were poured into aluminum molds and gelation was thermally induced at 80°C for 20 minutes in a heated press to obtain the desired thickness. The gels were then cored to the desired diameter such that each scaffold was 10 mm in diameter and 1.57 mm thick.

Prior to use, all scaffolds were sterilized by submersion in 200-proof ethanol, performed under vacuum to drive the ethanol into inner pores. The scaffolds were kept in ethanol for 5 hours under agitation, with additional vacuum drawn twice during the process. All scaffold processing after this stage was performed in an aseptic environment with sterile materials and buffers. The sterile scaffolds were rinsed with 0.1 M phosphate buffered saline (PBS), and transferred to 25 mL of PBS in 50 mL centrifuge tubes. The tubes were placed under agitation, with PBS replaced with fresh solution after 2, 4, and 6 hours, and continued under agitation overnight. This procedure removed the ethanol from the scaffolds to allow for cell growth.
5.2.3. **In Vitro Culture**

Sub-confluent MC3T3-E1, subclone 4 (ATCC, Manassas, VA) mouse preosteoblasts were statically seeded onto the sterile WPI scaffolds to begin each time study. Complete growth medium used in this study was ascorbic acid free, α-modified essential medium supplemented with 10% fetal bovine serum, 10,000 units/mL penicillin-streptomycin, and 25 μM amphotericin B, all obtained from Invitrogen (Carlsbad, CA). All cells used were passage 10 or lower. The cells were removed from the culture flask with Trypsin-EDTA, then pelleted by centrifuge. The supernatant was removed and the cell pellet was resuspended in fresh growth medium.

The cell suspension (20 μl of $10^5$ cells/mL) was pipetted onto each scaffold surface and incubated for one hour at 37°C in a 5% CO$_2$ atmosphere with 99% humidity to allow cells to adhere. Each scaffold was then rinsed with fresh medium to remove unadhered or loosely adhered cells, and supplied with 1 mL of fresh medium per scaffold. The seeded scaffolds were maintained in the wells of non-tissue-culture-treated 24-well plates. The cells were grown in complete growth medium for 1, 3, 5, 7, 14, or 21 days. Fresh medium was supplied every three days in culture.

5.2.4. **Mineralization Studies**

Samples used in mineralization studies were seeded with cell suspension, 100 μl of $10^4$ cells/mL, and cultured in complete growth medium, supplemented with 50 μg/mL ascorbic acid to induce differentiation and 3.0 mM Na$_2$HPO$_4$ to provide a source of phosphate. The scaffolds were cultured for 28 days, with fresh medium supplied every three days.

5.2.5. **Fluorescence Microscopy**

At the end of each incubation period, the *in-vitro* cultured scaffolds were rinsed with PBS, then fixed with 4% paraformaldehyde solution and permeabilized with 0.1% Triton X-100. Following
fixation and permeabilization the nuclei were stained with Hoescht 33258 (Invitrogen, Carlsbad, CA) and the samples were rinsed and placed in PBS in preparation for viewing. No viability stain was employed, as several rinses with PBS prior to fixation were assumed to remove non-viable cells loosely associated with the scaffolds.

The samples were imaged by a Nikon Microphot-FXA fluorescent stereomicroscope at 10X magnification at 14 blindly-selected locations per sample, and the nuclei visible within the viewing window were counted. Images were enhanced using ImageJ for Microscopy software to increase the contrast between the signal and the background, and to suppress the light from non-specific binding of the dye (as this study was to qualitatively label the nucleus). For representative image of raw data see Appendix D.

Cell density was calculated by first dividing each cell count obtained by the known area of the viewing window. The mean of all values obtained for each sample was used as a single measurement, and at least three such independent repetitions were carried out for each data point.

### 5.2.6. Curve Fitting

The cell densities obtained from the fluorescence micrographs were converted to total cell numbers by multiplying by the surface area of each scaffold, assuming a perfectly circular two-dimensional surface. Only the top surface of each disc-shaped scaffold was considered, since the bottom surfaces of several scaffolds were viewed and shown to contain no adhered cells. The cell numbers obtained were plotted versus incubation time, and fit to each of two equations. Data for 0-14 days in culture were fit to an exponential growth model (Eq. 5.1), and the full data sets were fit to the Gompertz model (Eq. 5.2), which captures the saturation behavior characteristic in the proliferation of cells on a finite growing surface in the form of a retardation constant.
\[ N(t) = N_0 \exp(kt) \quad \text{(Eq. 5.1)} \]

\[ N(t) = N_0 \exp \left( \frac{k_+}{k_-} \left( 1 - e^{-k_-} \right) \right) \quad \text{(Eq. 5.2)} \]

N(t) is the cell density (cells per unit area) as a function of incubation time, t, with \( N_0 \) representing the initial cell density. The exponential growth constant is represented by \( k \) in Eq. 5.1 and \( k_+ \) in Eq. 5.2, and \( k_- \) in Eq. 5.2 is the retardation constant.

SAS software was used to perform the non-linear regression. The regressions were completed using Levenberg-Marquardt minimization algorithm and tested for sensitivity to initial guess. Only stable solutions with regard to initial guess were considered reliable fits. Stability is defined as a solution shown to be independent of the initial guess values within a reasonable range. Parameters extracted from these regressions were used to characterize the contributions of the various scaffold components to the scaffold performance in supporting the adhesion and proliferation of osteoprogenitor cells.

### 5.2.7. Scanning Electron Microscopy with Energy Dispersive X-Ray Spectroscopy

Mineralization samples were imaged using an FEI Quanta 200 scanning electron microscope (SEM) coupled with energy dispersive x-ray spectroscopy (EDS) for elemental analysis to elucidate the extracellular nature of the culture. Upon reaching the end of their incubation, the samples were each rinsed with PBS, fixed with 2% gluteraldehyde/1% formaldehyde in 0.1 M PBS. The samples were then post-fixed with 0.1 M cacodylate buffer with 0.004 M glycine, dehydrated in a graded series of ethanol and dried by critical point CO\(_2\). Immediately prior to imaging, the samples were mounted to SEM stages and sputter-coated with platinum. Samples were imaged under high vacuum at 10 kV or 15 kV for image capture and 15 kV for EDS analysis.
5.2.8. Statistical Analysis

At least three repetitions were used for each scaffold composition, with 14 images for each scaffold, similarly distributed about the scaffold to account for radial changes in cell density, but blindly selected and captured (n ≥ 42). The convergence criterion for the objective function was set to $1 \times 10^{-4}$ in all regressions. The Wald test in SAS was used to calculate 95% confidence intervals corresponding to regression parameters.

5.3. Results and Discussion

5.3.1. Direct Cell Count

In this study, a direct approach was used to quantitate the effect that each gel component had on the overall behavior of the tissue scaffold. Viability and proliferation studies generally found in the literature are either qualitative or spectroscopic, without a conversion factor relating the optical values back to an explicit number of cells. Conversely, in this study, discrete cell counts were performed of cells as they were in their native state. We have found this to be a more reliable approach to truly probing the experimental system. Other studies have used direct cell counts on histological sections from \textit{in vivo} experiments to test the effect of a systemic stimulus [12], performed discrete counts of cells grown in a tissue-culture flask [13], or performed a discrete count of cells on a scaffold, but went to great lengths to ensure complete cell removal and qualitatively assessed the proliferation rates [14]. Presented here is the only use of fluorescent direct cell counts for rigorous calculation of total cell numbers on bone scaffolds to date. From previous experiments, viable cells on the scaffolds were expected, but it was hypothesized that the differences between scaffolds would be subtle. Therefore, the comparison of the kinetic parameters associated with the cell growth was used to shed light on these differences in an objective manner.
5.3.2. Non-Linear Regression

Two different functions were used to model the growth since two distinct growth regimes are observed for adherent cells on a finite growth surface. So long as space and nutrients are ample, cell proliferation proceeds exponentially. This behavior characterized the system at short incubation times and low cell densities, and was modeled by a simple exponential growth model. However, as space becomes limited, intercellular communication inhibits cell division, resulting in longer doubling times and a deceleration in growth. This behavior characterized the system at long incubation times and high cell densities, and was modeled by the Gompertz function. This relation, previously used to model aging and mortality [15], concentration-effect relationships in pharmacology [16], and mammalian cell proliferation in tumorigenesis [17], does not describe initial growth as well as the exponential growth model, but captures the deceleration in the latter saturation regime inherent to the system, whereas the exponential function is quick to diverge.

The cell density data for incubation times up to 14 days were plotted and fit to an exponential growth model. The data and exponential fits for varying concentrations of WPI, CaCl$_2$, and amylopectin are shown in Figs. 5.1, 5.2, and 5.3, respectively. The full set of cell proliferation data (21 days) was plotted and fit to the Gompertz function. These data and regression curves are shown for varying concentrations of WPI, CaCl$_2$, and amylopectin in Figs. 5.4, 5.5, and 5.6, respectively. The Gompertz function accurately described the growth pattern of cultures on most of the scaffolds tested.

5.3.3. Seeding Efficiency

The scaffolds in the study underwent static seeding with a cell suspension of 2,000 cells for one hour. The seeded scaffolds were rinsed with fresh media prior to incubation. The fraction of cells that remained adhered following the rinse was defined as the seeding efficiency. For each set of
conditions, this parameter, $N_0$ in Eq. 5.1, was calculated from the non-linear regression of the data to the function describing exponential growth. The values for seeding efficiency onto each tested surface are listed in Table 5.1.

The cells adhered with high efficiency to the majority of scaffold surfaces – greater than 70% for two thirds of the tested compositions, with one third displaying greater than 80% efficiency. Additionally, scaffold composition was shown to impact the seeding efficiency. Scaffolds with low WPI content did not effect seeding as well as the higher-WPI scaffolds. At low WPI concentrations, suspended cells were unable to quickly adhere to the scaffold surface. However, with WPI concentration of 35% w/v or greater, seeding efficiency markedly increased and remained roughly constant. These higher-WPI scaffolds are stiffer than their lower-WPI counterparts – a property known as favorable to osteoblast adhesion [18]. The improvement
Table 5.1 – Non-Linear Regression Parameters Describing Osteoblastic Proliferation on WPI Scaffolds.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>SE (95% CI)</th>
<th>k (95% CI)</th>
<th>k (95% CI)</th>
<th>k (95% CI)</th>
<th>N∞ (cells/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W20/C10/A0</td>
<td>0.36 (0.10 to 0.63)</td>
<td>0.259 (0.206 to 0.312)</td>
<td>2.07 (0.00 to 4.30)</td>
<td>0.214 (0.000 to 0.446)</td>
<td>55600</td>
</tr>
<tr>
<td>W25/C10/A0</td>
<td>0.24 (0.06 to 0.43)</td>
<td>0.281 (0.227 to 0.336)</td>
<td>1.63 (0.00 to 3.27)</td>
<td>0.177 (0.000 to 0.354)</td>
<td>69000</td>
</tr>
<tr>
<td>W35/C10/A0</td>
<td>0.85 (0.76 to 0.93)</td>
<td>0.221 (0.214 to 0.229)</td>
<td>0.623 (0.420 to 0.826)</td>
<td>0.112 (0.000 to 0.149)</td>
<td>148000</td>
</tr>
<tr>
<td>W40/C10/A0</td>
<td>0.71 (0.39 to 1.00)</td>
<td>0.259 (0.226 to 0.291)</td>
<td>1.10 (0.27 to 1.92)</td>
<td>0.151 (0.000 to 0.264)</td>
<td>159000</td>
</tr>
<tr>
<td>W45/C10/A0</td>
<td>0.78 (0.68 to 0.89)</td>
<td>0.256 (0.246 to 0.266)</td>
<td>1.78 (0.48 to 3.08)</td>
<td>0.203 (0.000 to 0.352)</td>
<td>118000</td>
</tr>
<tr>
<td>W35/C0/A0</td>
<td>0.97 (0.76 to 1.00)</td>
<td>0.186 (0.170 to 0.203)</td>
<td>0.351 (0.232 to 0.471)</td>
<td>0.071 (0.000 to 0.095)</td>
<td>208000</td>
</tr>
<tr>
<td>W35/C5/A0</td>
<td>1.31 (0.72 to 1.89)</td>
<td>0.163 (0.129 to 0.198)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>W35/C10/A0</td>
<td>0.72 (0.52 to 0.92)</td>
<td>0.223 (0.203 to 0.244)</td>
<td>0.589 (0.256 to 0.921)</td>
<td>0.105 (0.000 to 0.165)</td>
<td>148000</td>
</tr>
<tr>
<td>W35/C15/A0</td>
<td>0.74 (0.58 to 0.89)</td>
<td>0.195 (0.179 to 0.211)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>W35/C20/A0</td>
<td>0.50 (0.40 to 0.60)</td>
<td>0.224 (0.209 to 0.240)</td>
<td>0.463 (0.323 to 0.602)</td>
<td>0.078 (0.000 to 0.102)</td>
<td>212000</td>
</tr>
<tr>
<td>W35/C10/A10</td>
<td>0.81 (0.00 to 1.00)</td>
<td>0.276 (0.113 to 0.439)</td>
<td>0.447 (0.282 to 0.612)</td>
<td>0.104 (0.065 to 0.142)</td>
<td>112000</td>
</tr>
<tr>
<td>W35/C10/A15</td>
<td>0.65 (0.53 to 0.77)</td>
<td>0.335 (0.307 to 0.364)</td>
<td>0.738 (0.629 to 0.848)</td>
<td>0.150 (0.128 to 0.173)</td>
<td>95800</td>
</tr>
<tr>
<td>W35/C10/A20</td>
<td>0.63 (0.43 to 0.83)</td>
<td>0.328 (0.278 to 0.378)</td>
<td>0.684 (0.614 to 0.755)</td>
<td>0.131 (0.118 to 0.145)</td>
<td>130000</td>
</tr>
<tr>
<td>W35/C10/A25</td>
<td>0.77 (0.00 to 1.00)</td>
<td>0.333 (0.042 to 0.624)</td>
<td>0.811 (0.380 to 1.243)</td>
<td>0.154 (0.072 to 0.236)</td>
<td>126000</td>
</tr>
</tbody>
</table>

a Expressed as W(% w/v WPI)/C(mM CaCl₂)/A(% of WPI weight)
b Seeding efficiency, or fraction of adhered cells out of total seeded cells
c 95% confidence interval by the Wald test
d Exponential growth constant based on cell density data for 14 days, fit to the exponential growth model
e Exponential growth constant based on cell density data for 21 days, fit to the Gompertz growth model
f Rate of retardation constant based on cell density data for 21 days, fit to the Gompertz growth model
g Theoretical scaffold saturation limit, predicted by the Gompertz growth model as time approaches infinity
h Anomalous value of efficiency, calculated as greater than 100%. Should be considered only qualitatively, in comparison to surrounding values
i Missing values for scaffolds for which convergence could not be reached for the Gompertz function
follows the relationship previously reported for the mechanical properties of this scaffold [7].

Strengthening this conclusion is the observed dependence of the seeding efficiency on CaCl₂ concentration; it mirrors previously reported results for gel stiffness with addition of CaCl₂ [19].

Amylopectin was not definitively found to impact the seeding efficiency, but the data nonetheless suggest an inverse relation between amylopectin concentration and seeding efficiency – also supported by the response of N₀ from the Gompertz model. Over the span of concentrations tested, the seeding efficiency values spanned 30%. The cause for the decreased efficiency is not believed to stem from the molecular structure of amylopectin. The structure contains multiple hydroxyl groups which should promote seeding. It is therefore more likely that it simply does not promote seeding as effectively as WPI, and the competitive effect creates the observed trend. A higher surface concentration of amylopectin leads to a lower concentration of
functional groups inherent to WPI that enhance cell seeding, such as carboxylic acids, primary amines, or any adhesion moiety yet unknown. These seeding efficiencies exceeded those reported for seeding of scaffolds of collagen/hydroxyapatite (HA) [20] or PLA/calcium phosphate [21], and were achieved under more stringent seeding conditions, and comparable to those reported for centrifugal seeding of poly (ε-caprolactone-co-L-lactide) scaffolds [22].

5.3.4. Exponential Growth Rate Constant

The growth rate constants (k in Eq. 5.1) were obtained from fitting the data to an exponential growth model and are listed in Table 5.1. Based on the model, scaffold composition had little effect on the growth rate constant. Changing the WPI content had no impact on the growth rates on the scaffolds. WPI content was not expected to affect the growth rate, since the local environment detected by each cell could be considered saturated with WPI and therefore

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Fig. 5.5 – Gompertz model growth curves for varying scaffold CaCl₂ concentration. Proliferation data for MC3T3-E1, subclone 4 cells cultured for 1-21 days on scaffolds containing 35% w/v WPI and 0% amylopectin and varying in CaCl₂ concentration. Curves represent regressed fits of the data to the Gompertz growth model. Dotted lines represent the best fit for data sets with unstable solutions.

Fig. 5.6 – Gompertz model growth curves for varying scaffold amylopectin concentration. Proliferation data for MC3T3-E1, subclone 4 cells cultured for 1-21 days on scaffolds containing 35% w/v WPI and 10 mM CaCl₂ and varying in amylopectin concentration. Curves represent regressed fits of the data to the Gompertz growth model.
constant – resulting in a relatively constant growth rate. Likewise, the rate constant varied little with respect to CaCl₂. Although some upward variation was observed, a distinct trend could not be determined with statistical certainty. Calcium chloride added to the matrix increases surface roughness on the micrometer scale [7], likely to promote adhesion. The results of the study of amylopectin concentration were more complex. The comparison among the different concentrations could not be made, as four of the five scaffold concentrations tested exhibited a departure from exponential growth after only seven days – allowing only four data points to be used for the model regression. However, the highest-concentration data (0.25 g/g WPI) could be fit reliably to the 14-day data but not to the 7-day data. Because the true departure from exponential growth occurs somewhere between the two, the parameters extracted from the different fits could not be directly compared. Thus, conclusions regarding the effects of amylopectin on cell proliferation were based on the assessment afforded by the full-range fit to the Gompertz model.

5.3.5. Gompertz Function Rate Constants

The fit to the Gompertz function was valuable in determining primarily two parameters – the growth rate constant, k⁺, and the rate of retardation constant, k⁻. The remaining parameter included in the model – representing the initial cell density – was not used. After 21 days in culture, 13 of the 15 scaffolds showed an approach to scaffold saturation, and decelerating cell proliferation. Two curves could not be fit to the Gompertz model, since they were still primarily characterized by exponential growth, and there was insufficient data to converge on a single solution for the saturation value of the scaffold. The outcome merely indicates that over 21 days, proliferation of these cultures is still well characterized by the exponential function. For the plotted results of the exponential fit to the two data sets see Appendix D.
The growth rate constant $k_+$ and rate of retardation constant $k_-$ obtained for each set of conditions are listed in Table 5.1. The growth rate constant, $k_+$, represents a parameter similar to the rate constant obtained from the exponential growth fit. For WPI as well as for CaCl$_2$, no trend could be established for this parameter. While an observational trend occurred when WPI was varied, with a minimum rate at 35% w/v WPI, it could not be validated statistically. Two of the scaffold compositions in the calcium chloride data set still exhibited exponential growth at the end of the experiment, and could not be fit to the Gompertz function, leaving insufficient data to establish a trend. As amylopectin concentration was increased, the data suggest an increased growth rate, but the trend could not be confirmed beyond the model uncertainty. The phenomenon may be a result of the added nutrient source available to proliferating cells. This branched carbohydrate is composed of glucose monomers and can be hydrolyzed to release glucose molecules for consumption. Increased temperature, as during thermal gelation, causes partial hydrolysis of amylopectin and increases its susceptibility to enzymatic degradation [23].

For all compositions, the response of $k_-$ to composition was qualitatively similar to that of $k_+$. The uncertainty in the regression parameters obscured any trend with respect to WPI. As with $k_+$, the three regressions were insufficient to establish a trend for $k_-$ with respect to CaCl$_2$ concentration. With increased amylopectin concentration in the scaffolds, the overall suggested trend was an increase in the value of $k_-$. For each growth curve, the limit of the Gompertz function as time approaches infinity was calculated. The value of the limit corresponds to a theoretical saturation cell density on the scaffold. These values are listed as $N_\infty$ in Table 5.1. While varied, the theoretical saturation densities did not exhibit any compositional dependence.

It is worth noting that although the data were well described by the function, yielding precise fits, most confidence intervals precluded a confirmation of observed trends. The most likely principal
cause for the uncertainty is the number of data points available for the fit. This is especially
evident when the confidence intervals for \(k_+\) and \(k_-\) are compared. The former shows much
narrower confidence intervals than the latter across the compositional range, and it is clear that
there are more data points characterized by the exponential growth portion of the model than
those describing the subsequent deceleration.

For all tested compositions – spanning the full possible compositional ranges – the cultures
thrived upon the scaffold surface and exhibited exponential growth for as long as growth area
was available, and increasing cell numbers for the length of the study.

5.3.6. Scaffold Mineralization

It is shown in the representative micrograph in Fig. 5.7a that after 28 days in culture, the
scaffolds were fully confluent, and that the cells had differentiated and begun to lay down a
mineralized matrix. The x-ray microanalysis showed the prevalent calcium and phosphorus
content, indicating a calcium phosphate phase over the scaffold (Fig. 5.7d). It was likewise found
that acellular scaffolds cultured under the same conditions developed a calcium phosphate layer
on the surface (Fig. 5.7b, e), which points to a pre-treatment possibility. When a non-
differentiating subclone of MC3T3-E1 cells was cultured under the same conditions, no calcium
phosphate phase could be detected, although the culture itself thrived and fully populated the
surface (Fig. 5.7c, f).

The analysis of the scaffolds containing 0 mM \(\text{CaCl}_2\) makes evident that this matrix component
actively provides the calcium necessary for mineralization, and the nominal 1.8 mM \(\text{Ca}^{2+}\) ions
present in the mineralization medium is not sufficient for bone formation. No additional calcium
source was provided in this study in order to elucidate this contribution. The cells grown on the
scaffold did not produce any mineral layer, and no calcium phosphate precipitation took place on
the surface of the acellular scaffold (Fig. 5.8a, b, e, f). As expected, no calcium phosphate was found on the scaffold surface with the non-mineralizing subclone. When high levels of calcium were incorporated into the scaffold, extensive mineralization resulted on the acellular scaffolds (Fig. 5.8d, h), though mineralization on the cell growth surface was limited (Fig. 5.8c, g).

The effects of the use of amylopectin as a scaffold additive on the mineralization process are summarized in Fig. 5.9. No adverse impact was found, and by qualitative analysis, scaffold mineralization was carried on uninhibited. High levels of calcium and phosphorous were found on acellular scaffolds of both low (Fig. 5.9b, f) and high amylopectin concentrations (not shown), and for the differentiating-cell-populated scaffolds of both compositions (Fig. 5.9a, c, e, g).
again, the scaffolds cultured with the non-mineralizing subclones showed only trace concentrations of calcium and phosphorous (Fig. 5.9d, h).

This study marks the first bone-formation event on the surface of a whey protein gel. The results show that the proposed scaffold material supports the formation of a mineralized scaffold, and implicitly, the differentiation of the model preosteoblastic cell line into osteoblasts. Notably, mineralization occurred at nominal calcium ion levels – without added calcium above that already present in the complete growth medium. It is therefore likely that the calcium from the scaffold matrix itself can be drawn upon in order to form a calcium phosphate phase.

Culturing scaffolds that contained 0 mM CaCl$_2$ in mineralization conditions proved that these nominal calcium ion levels – 1.8 mM CaCl$_2$ present in the growth medium – are sufficient to
enable active mineralization by differentiated osteoblasts but not mineralization by deposition onto acellular scaffolds, although mineralized areas were more prevalent on calcium-containing scaffolds. The scaffolds found successful in this study achieved a level of cellular mineralization comparable to that obtained for poly(ε-caprolactone) [24], collagen, and collagen/HA [25] scaffolds, known for their potential in bone regeneration.

The presence of precipitated calcium phosphate on acellular scaffolds could have been expected. The incubation at cell-culture conditions is sufficiently similar to the treatment with simulated body fluid, designed into many fabrication techniques to increase the bioactivity of a scaffold surface, commonly used to treat titanium implants [26], poly (L-lactic acid) [27], and silica glass scaffolds [28]. This phenomenon was common to all scaffolds that contained calcium chloride.
The concentration of WPI in the scaffold did not exhibit a qualitative difference in achieving mineralization on the scaffold surface. It is possible that a time study and a rigorous quantitative analysis would reveal a difference in the promotion of mineralization by the varying scaffold compositions. An encouraging result was that mineralization was found to proceed uninhibited in the presence of amylopectin in the gel matrix. It’s been previously found that increased levels of glucose are a factor leading to inhibited bone formation and remodeling, as found commonly among diabetic patients [29]. Amylopectin has several beneficial contributions to the properties of the scaffold-cell system, such as increased compressive strength and modulus and increased kinetic growth rate of cultured cells. It is therefore of great consequence that it presents no adverse impact on mineralization – the ultimate goal for the system. The apparent lack of mineralization on the surface of the high-CaCl$_2$ scaffold is characteristic of most spots probed on the surface. However, several bone nodules were found upon the scaffold (See Appendix D).

A quantitative x-ray microanalysis was performed for every EDS spectrum collected. However, due to limitations inherent to the technique, the resulting data were used merely as a guideline in assessing calcium and phosphorous levels, and in order to deconvolute the overlapping phosphorous $K\alpha$ peak at 2.010 keV and the platinum $M\alpha$ peak at 2.047 keV, present due to sputter coating of the samples. The necessity for this was made clear by analyzing the spectrum in Fig. 5.8e, which represents the surface of a cell on a CaCl$_2$-free scaffold. While the shared P-Pt peak appears large, the contribution of phosphorous was 40% of the peak. Scrutiny of the spectra in Fig. 5.7-5.9 allows the reader to locate x-ray peaks that are not labeled or addressed thus far. These peaks correspond to elements resulting from sample preparation (e.g. arsenic) or other impurities introduced to the surface after sample fixation (e.g. zinc, bromine). While these peaks are not labeled, they were included in the initial quantitative analysis performed at each location and only represented trace concentrations.
The morphology of the acellular scaffolds varied with composition. The structures of scaffolds containing 0 or 10 mM CaCl$_2$ showed a highly regular linear pattern over the full range of the scaffold (Fig. 5.7b, 5.8b). This was attributed to the microscopic structure of the aluminum molds the gel was cast from. When CaCl$_2$ content was doubled, or when a high level of amylopectin was incorporated into the matrix, this pattern was no longer exhibited (Fig. 5.8d, 5.9b, respectively). The difference is believed to be the effect of the composition on the scaffold degradation rate. At 20 mM, the scaffold is below its peak strength [7], and less stiff than at lower salt concentration. Both could contribute to enhanced degradation. Amylopectin, on the other hand, increases both the compressive strength and modulus of the gel (results not shown), but it may cause irregularities in the matrix that promote degradation. This effect is presumed to be minor, as large-scale order is still observed.

The experiments in this study were performed in two dimensions, presenting a limitation when the target system to be modeled contains various three-dimensional features. The discrepancies between the geometries will require investigation using a system that closely mimics the natural implant environment. The results of this analysis do, however, demonstrate the suitability of WPI for use as a bone tissue regeneration scaffold based on the cellular-interaction characteristics required of such a device.

5.4. Conclusions

Cell density data were gathered for osteoblasts adhered to varying growth-surface compositions for 21 days in culture. The time-dependent cell density data were successfully fit to exponential growth and Gompertz sigmoidal growth models, and kinetic parameters were extracted from the models and compared to determine the range of scaffold compositions most suitable for osteoblast seeding and proliferation. All tested compositions supported the adhesion and
proliferation of preosteoblasts for the full 21-day study. Scaffolds with WPI concentration of at least 35% WPI and 0-10 mM CaCl₂ demonstrated enhanced seeding efficiency. Efficiencies ranged from 24% to 97% of cells initially in suspension. Growth rate constants remained roughly independent of scaffold composition, with a possible increase in rate for higher CaCl₂ and amylopectin concentrations. The rate of retardation constant, or saturation of the scaffold, appeared independent of scaffold composition.

The mineralization ability of acellular scaffolds and populated scaffolds was qualitatively tested for compositional dependence. Calcium and phosphorous were detected in all scaffolds containing CaCl₂ in the matrix, indicating the formation of a calcium phosphate layer on the surface. Pre-incubation in simulated body fluid was not required for the deposition to occur. When MC3T3-E1 cells were cultured atop the scaffolds for 28 days, saturation was reached in most scaffolds. The desirable morphology of cells, extracellular matrix, and mineralization were observed in all cases, though only sporadic mineralization was observed on scaffolds containing 0 mM CaCl₂. Scaffolds cultured with a non-mineralizing subclone of the cell line served as a negative control and exhibited no evidence of calcium phosphate formation. See Appendix E for additional information on cell morphology.

It was concluded from this study that WPI-based gels have great potential for use as a bone tissue regeneration scaffold, and that proliferation and mineralization studies preclude no composition in the available range, though high WPI, low CaCl₂, and high amylopectin concentrations were found to produce the most favorable cellular response. Since the same combination was previously found to produce the best mechanical properties within the range of compositions, little decision-making is necessary and a robust optimum composition has been established for the system.
5.5. References


[29] Ogawa N, Yamaguchi T, Yano S, Yamauchi M, Yamamoto M, Sugimoto T. The combination of high glucose and advanced glycation end-products (AGEs) inhibits the mineralization of osteoblastic MC3T3-E1 cells through glucose-induced increase in the receptor for AGEs. Hormone and Metabolic Research. 2007;39:871-5.
Chapter 6: Pore Structure Analysis of Whey Protein Isolate Gels by X-Ray Microcomputed Tomography

6.1. Introduction

Porosity and pore network characteristics are among the most important properties in tissue engineering for dictating the level of success of a scaffold [1-3]. They dictate the uniformity of the initial seeding of scaffolds with cells [4], govern the flow and diffusion of nutrients to proliferating cells and of cell metabolites [2], affect the degree of migration of cells into the core of the scaffold and their survival therein through vascularization [5-6], as well as determine the final structure of the tissue that is formed [7]. However, characterization of three-dimensional scaffolds poses a great challenge for conventional porosity techniques such as mercury intrusion porosimetry and microscopy methods. Scaffold materials, though robust, are often unable to withstand the pressures exerted by intrusion porosimetry, so the pressures can collapse the sample before the full set of data is collected [8]. For hydrogels or other swelled or hydrated structures, methods for porosity and pore network determination that require a dry sample are incompatible, because such samples tend to shrink on drying. Thus the test represents information on a pore network that is different from the true, or hydrated, structure. The technique is also limited to the surface-accessible pores, excluding isolated pores from the analysis. Furthermore, heterogeneity may exist in the sample as an artifact of processing or deliberately designed into the scaffold structure [9-10]. Bulk characterization techniques are unable to capture this pore-scale information [8].

The technique of x-ray microcomputed tomography (XMCT) has been rapidly gaining popularity in the field of tissue engineering because of its clear advantages over conventional methods [8,
To date, XMCT has been used primarily to obtain bulk properties of a matrix [12-15] without taking advantage of the information available for location-specific characteristics of the material of interest. By combining the microcomputed tomography technique with modeling tools to translate the three-dimensional voxel information into a pore-interconnect map, one can precisely quantify scaffold characteristics. These characteristics may then be used to objectively compare material and processing variables, both to one another and against the ideal network structure. Such comparisons will aid in decision-making, process optimization, and quality assessment.

The analysis technique presented here is illustrated by applying it to a specific case study. In this study, whey protein isolate (WPI) gels of different concentrations are analyzed for porosity and pore network structure. Based on some promising previous results, WPI is being considered as an emerging scaffold material for bone regeneration [16-17]. The naïve gel relies solely on the presence of air in the precursor suspension to create porosity in the material. It then becomes necessary to determine the nature of the network created. This experimental system is used to demonstrate the utility of XMCT for obtaining bulk as well as spatial distributions of scaffold properties. This is a more robust set of information than that generally gleaned from the technique.

6.2. Materials and Methods

6.2.1. Materials

All water used in this work was >18 MΩ water from a Direct-Q 3 water purification system (Millipore, Billerica, MA). The materials used were WPI powder from Davisco Foods International (Eden Prairie, MN), used as provided, and calcium chloride dihydrate and sodium azide from Mallinckrodt Chemicals (Hazelwood, MO).
6.2.2. Sample Fabrication

Three samples were prepared as previously described [16]. Briefly, WPI powder was added to an aqueous CaCl$_2$ solution of half the volume and double the concentration desired for the final mixture. Sodium azide, 0.2%, was added to the suspension as a preservative. The mixtures were each vortexed to mix and allowed to equilibrate overnight. They were then adjusted to volume for the final target concentrations of 20%, 35%, and 45% w/v WPI by adding water and gently agitating. The precursor suspension was cast into cylinders (L = 7.62 cm, D = 10 mm) and gelation was thermally induced by curing at 80°C for 60 min. The solid gels were cooled at room temperature and kept in a moist, sealed environment at room temperature to maintain the gel structure until testing. The three gel samples are hereafter referred to as 20WPI, 35WPI, and 45WPI.

6.2.3. Microtomography

X-ray microfocus computed tomography is an imaging technique that enables the creation of high-resolution three-dimensional images of opaque materials within which the structure is geometrically complex. In this system, x-rays are created in an x-ray tube and propagated towards and through a sample. Some of the x-rays that pass through the sample are collected by the image intensifier and converted into images. In the case presented here, high resolution images were created that are comprised of cubic voxels (or 3D pixels), which were 14.59 µm on an edge.

During the scanning, x-rays are propagated towards and through the sample, which rotates 360°. During the rotation, x-ray attenuation data are collected at 2400 sample angles. This effectively divides the sample material into small, highly-resolved sample elements, or voxels. X-ray attenuation is determined by the material properties, specifically the sample density and atomic
number in the area of the voxel. Following each rotation, in order to capture the full length of the sample material, the XMCT sample stage moves vertically, in very precise increments, to a new sample elevation. Once the new sample elevation is obtained, the x-ray attenuation data are again collected. The process of collecting x-ray attenuation data during a sample rotation and moving the sample vertically is repeated until the desired length of the sample material has been scanned by x-ray. The x-rays that pass through the sample and are projected onto the image intensifier are analyzed and converted to two-dimensional grayscale (16-bit) images. Because the 2D images that are produced are perfectly registered along the length, or z-axis, of the material, a 3D image is readily obtained by stacking the slices.

Once the 3D image is constructed, the image is manipulated to reduce artifacts associated with x-ray attenuation data and to simplify the image. The first artifact to remove is beam hardening. This occurs in x-ray systems that use a polychromatic source. This polychromatic energy is attenuated differently depending upon the energy of each x-ray. Lower energy x-rays are attenuated along the edges of the sample and only the higher energy x-rays pass through the entire sample diameter. To correct for this differential attenuation, or beam hardening, an algorithm is used to normalize the voxel image values so that the exterior of a homogenously dense material, which appears denser in the initial image, is corrected to have the same or similar voxel values as the same material on the sample interior.

Once this beam hardening correction is complete, the image is divided into regions of interest. The regions of interest in these samples are the whey protein matrix and void space. To differentiate these two, an indicator kriging algorithm was used to isolate the voids from the matrix [18]. This process assigns voxels in the image into two distinct populations – matrix material and void space – and then, in cases where the voxels cannot easily be assigned to one
population or the other, typically the areas at the pore edges, voxels are assigned based upon the voxel values in the local neighborhood. In this case, a fairly narrow range of voxel values were corrected based upon the neighborhood. Voxels with a grayscale value of less than 6690 are automatically assigned to the pore population, voxels with values of 6690 to 6710 were determined by the neighborhood voxels, and voxels with values greater than 6710 were assigned to the matrix material (Fig. 6.1). The resulting image is a 1-bit grayscale image where the pores are comprised of zero-valued voxels and the matrix (whey protein) is comprised of voxels with a value of 1 (Fig. 6.2). XMCT data were collected, processed, and segmented by Dr. Allen H. Reed at the Naval Research Laboratory, Stennis Space Center.

6.2.4. Network Modeling

A pore network structure was generated from the segmented volume data using an in-house algorithm called vox2net. It is based on algorithms designed for granular materials [19]. However, instead of operating from a known granular structure, it operates directly on the voxel image and therefore can be used for a wider range of structures (foams, fibrous materials, etc.).

As with the grain-based algorithm, each pore was characterized by a maximal inscribed sphere (i.e., a hypothetical sphere that is constrained from both movement and growth by its contact
with surrounding solid phase). In all cases, the maximal inscribed spheres were found using the same nonlinear optimization process used with the grain-based algorithm. However, because this optimization procedure is computationally expensive, a number of different schemes were devised to define subsets of seed voxels from which to run the optimization. Presently, three approaches can be used:

1. Perform a voxel burn or erosion process [20] in the void phase to define voxels that are local maxima in the distance function (from the surface). These are viewed as likely locations for pore centers.

2. Use all void-phase voxels as potential seeds for the nonlinear optimization, but eliminate all voxels inside a maximal inscribed sphere once it is found, on the premise that other seed locations would likely converge to the same maximal inscribed spheres. Because these spheres occupy a significant fraction of the void volume, this approach is dramatically faster than using every voxel as a seed.

3. Use all void-phase voxels as seeds for the nonlinear optimization. This approach is slow but comprehensive.

In this work, option 1 was used because the pores are typically spherical, which means they can easily be identified by the voxel burn process.

Once the centers of pores were located (i.e., the centers of maximal inscribed spheres), voxels surrounding each central location were collected using a modified watershed algorithm, which is designed to force the boundaries of two neighboring pores to intersect at the tightest constriction. This process resulted in every voxel in the data set being tagged with the pore number to which it belongs. It also allowed for the straightforward computation of geometric parameters (pore
volume, surface area, inscribed radius, etc.) simply by assembling the relevant set of voxels and performing voxel-based geometric operations on the set. Connectivity of the network was determined by searching for neighboring voxels that have different pore numbers. The resulting pore structure of each sample was stored as a .psn file (pore scale network), which is a data format developed and used by Prof. K.E. Thompson’s research group. Radial distributions of properties were calculated by dividing the voxel map of each sample into annular segments of equal thickness and averaging the properties over each annulus. The resulting values were plotted versus dimensionless radius. Algorithm selection, writing of the program code for construction of the pore structures and the computation of geometric parameters were performed by Prof. K.E. Thompson, Cain Dept. of Chemical Engineering, Louisiana State University.

Fig. 6.2 - 3D rendered images of (a) 20%, (b) 35%, (c) 45% w/v WPI gels, and (d-f) their respective ball-and-stick pore network representations.
6.2.5. Viscometry

Viscosities of WPI suspensions from 20% to 45% w/v WPI in water were measured using a cone and plate rheometer (Rheometric SR-5000N, Piscataway, NJ) at shear rates ranging between 0.1 and 50 s\(^{-1}\), as appropriate for each suspension. The shear rates tested varied with suspension viscosity. The viscosity dependence on WPI concentration was used in subsequent calculations to describe the pore network structure derived from entrapped bubbles in the precursor suspensions. Viscometry experiments were performed by Prof. Kerry M. Dooley, Cain Dept. of Chemical Engineering, Louisiana State University.

6.2.6. Theoretical Threshold Viscosity

Bubbles are known to form during preparation of WPI precursor suspensions. The solution for a fluid sphere rising through a fluid medium was applied to a reference bubble of 100 μm diameter \{Happel, 1973 #275\}. In this analysis, the bubble was assumed to be in equilibrium, with the drag force, buoyant force, and gravitational force summed to zero (Eq. 6.1). The velocity used in the analysis was 1.27 m·s\(^{-1}\), the fastest applicable velocity corresponding to the height of the mold divided by the time until the onset of gelation (approximately 10 minutes). The density of the WPI suspension was assumed equal to the density of water, which is a close approximation.

\[
\rho_2 V_1 g + m_1 g + 4\pi \eta_{\text{crit}} \frac{D}{2} v = 0 \quad \text{(Eq. 6.1)}
\]

The bubbles are assumed to be spherical and unaffected by one another (flow in an infinite medium). Under these conditions, the highest Reynolds number in any system is \(1.59 \times 10^{-3}\), thus low enough for the use of this expression.

The force balance (Eq. 6.1) on the bubble was arranged to solve explicitly for the critical viscosity (Eq. 6.2) to entrap a 100 μm diameter bubble for the length of time until onset of
gelation. The viscosity value was compared to those of the precursor suspensions used for scaffold fabrication.

\[ \eta_{\text{crit}} = \frac{\rho_2 V g - m_1 g}{4\pi \frac{D}{v}} \]  
(Eq. 6.2)

6.3. Results

6.3.1. Porosity

Three WPI gel samples were analyzed by XMCT and a full 3D mapping of each sample was constructed. This voxel map included every pore within the solid and from it several key properties of the pore network were determined. The 3D representations of each solid and void content are shown in Fig. 6.2. Porosity showed strong dependence on WPI concentration (Fig. 6.3a). The greatest porosity achieved was 17.8%, observed for 45WPI, while only nominal void volume was observed for 20WPI. Additionally, the small void content found in 20WPI was primarily located in a cluster of large pores at the outer edge of the sample (Fig. 6.2a, d). The porosity was also evaluated for variation with respect to spatial position – both radially and axially within each sample. No characteristic of the pore network structure expressed any discernable trend along the axial direction (see Appendix F). However, for the two porous samples, the porosity was higher in the center of the cylindrical sample and decreased radially (Fig. 6.4a).

6.3.2. Interconnectivity

The pores in these scaffolds showed limited interconnectivity, as expressed by the pore coordination numbers (Fig. 6.3b), with the average coordination number observed in the 20WPI scaffold falsely high because of the previously-described inhomogeneity. In fact, the gel matrix exhibited higher interconnectivity with increased protein concentration. The radial profile of the
coordination number was roughly flat, revealing no spatial dependence of interconnectivity within the scaffold (Fig. 6.4b).

6.3.3. Pore and Throat Diameters

The concentration affected the inscribed pore diameters as well, but the differences in the mean pore size were relatively small – approximately 20 μm between each pair (Fig. 6.3c). However, because of the 20WPI scaffold irregularity, the mean pore diameters are falsely large. The effect is further clarified by examination of the pore size distributions (Fig. 6.5). The advantage of using XMCT for the determination of the pore size distribution is that the measurement is direct – every pore is counted and measured. Thus, the frequency of each pore size does not have to be
uncovered as a result of this analysis. It was evident that the number of pores was highly influenced by WPI concentration, with relatively few pores found in the 20WPI sample. This trend was consistent with the overall porosity measurement. Also, the shape of the distribution varied with WPI concentration. For low WPI, the distribution was highly positively skewed, with pores smaller than 30 μm occurring most frequently. For the intermediate concentration, 35% w/v WPI, the distribution was much closer to Gaussian, while for high WPI, the distribution was
once again positively skewed, but with most pores between 80 and 120 μm, and smaller pores less frequently observed. The latter distribution was also much wider, with a long tail, indicating a significant number of pores larger than 200 μm. Few pores greater than 200 μm were found in either of the other samples. However, against expectations, the median pore sizes of the two porous scaffolds (35WPI and 45WPI) were almost equal, both approximately 100 μm. Examination of the radial profiles of the pore diameters revealed that not only did the pore size vary radially within each sample, but the direction of the trend itself was affected by the protein concentration (Fig. 6.4c). At low WPI concentration, only small pores were observed within the core of the scaffold. The larger pores on the boundaries were an artifact of the inhomogeneity discussed above. At high WPI concentration, the largest pores were found in the scaffold center,
with average pore size decreasing with radial position. At intermediate WPI concentration, the pore size was roughly uniform, if slightly decreasing with increasing radial position.

The average throat diameter – diameter of the inscribed connections between pores – appeared roughly independent of WPI concentration (Fig. 6.3d). The two porous samples, 35WPI and 45WPI showed almost identical average throat sizes, and the average throat size for 20WPI is not a meaningful quantity, due to the localization of the pores on one narrow section of the sample (Fig. 6.2d). For the porous gels, the throat diameter was also uniform throughout each scaffold, or radially independent (Fig. 6.4d).

6.3.4. Viscometry

WPI suspensions, subjected to processing identical to that used for gelation samples, were tested at various shear rates and the viscosities measured at shear rates appropriate for each suspension (Fig. 6.6a). Of the six tested, the three more dilute suspensions exhibited Newtonian behavior, registering a constant viscosity with respect to shear rate. The three more concentrated suspensions demonstrated shear-thinning behavior, and a yield stress was observed for the two most concentrated suspensions, as typical of Bingham plastics. At a relatively low shear rate (averaged values between 10 and 11 s\(^{-1}\)), which is relevant to the analysis, the viscosity varied exponentially with WPI concentration, spanning almost three orders of magnitude (Fig. 6.6b). This behavior is typical of concentrated suspensions following Mooney’s equation at low ratio of volume fraction to packing factor [21-22].

6.3.5. Theoretical Threshold Viscosity

The viscosities of the precursor suspensions of the three tested WPI gels can be read from Fig. 6.6. Based on the drastic difference in pore size distribution of the 20WPI gel and the two others, a threshold viscosity behavior was assumed to dictate the pore network structure. The shift in
fluid type with concentration from Newtonian to non-Newtonian as observed in Fig. 6.6a supported this hypothesis. Known material properties and system parameters were used to solve Eq. 6.2 for the threshold viscosity that would enable a cast WPI suspension to entrap 100 μm bubbles within the gel matrix during gelation. This viscosity was calculated to be 0.64 P – a value eightfold higher than the measured viscosity of the 20% w/v WPI suspension, and an order of magnitude lower than the 35% w/v WPI suspension, thus further supporting the stated hypothesis (Fig. 6.6b).

6.4. Discussion

It is understood that the pores in the gel matrix originate from air bubbles in the gel precursor suspension that remain trapped during the curing of the gel. Therefore the observed pore network characteristics stem primarily from the fluid properties of the suspension. The suspension viscosity is an exponential function of WPI concentration (Fig. 6.6) and was found to influence
the pore network more than any other suspension property, and most observations can be attributed to it. The strong discrepancy between the 20% WPI gel and the two other gels suggested a threshold behavior, so it was hypothesized that the 20WPI precursor solution had too low a viscosity to trap bubbles prior to curing, while the viscosities of the 35% WPI and 45% WPI suspensions were sufficient to generate a drag force large enough to prevent bubbles from rising to the air-water interface. If the hypothesis is correct, there exists a threshold viscosity that is necessary and sufficient to prevent bubbles of the median diameter (approximately 100 μm) from rising to the interface within the time required for the sample to become solid (approximately 10 min). Moreover, the value of the threshold viscosity should lie between the measured viscosity of the 20% w/v WPI suspension and that of 35% w/v WPI.

Based on the assumptions and the rheological measurements of the WPI suspensions, a critical viscosity of 0.64 P was calculated. In addition to explaining the disparity between the structures formed from 20% w/v WPI and 35% w/v WPI suspensions, this result also explained the spread observed in the pore distributions of 35% WPI and 45% WPI, as bubbles larger than 100 μm can easily be supported by the drag forces imparted by the fluids.

The interfacial behavior of whey protein is another suspension property affecting the final pore network structure. WPI distributes preferentially into interfaces over bulk solutions [23]. The high-concentration suspensions used in this study all exceed the solubility limit of WPI [24] thereby driving excess protein to the air-liquid and PTFE-liquid interfaces. This behavior accounts for the recurring radial trends that exhibit a decrease in porosity or pore size outward towards the scaffold boundary.

The gels analyzed in this study are proposed as potential materials for use in bone tissue regeneration scaffolds. The type of pore network required for such scaffolds is fairly well
established. The scaffold must contain high porosity (void volume of at least 80-90%) [25]. The pores must be highly interconnected, with most or all of the inner porosity accessible from the scaffold surface [12]. The optimum mean pore size is a topic of some debate. It is accepted that it should be at least 100 μm [26-28], but perhaps greater than 300 μm in diameter [29]. Throat diameters of at least 100 μm improve initial seeding and cell viability in the center of a scaffold [30]. Based on these criteria, it is clear that in order to make the material suitable for use as a scaffold for tissue regeneration, more void volume must be incorporated into the matrix than what is available as a byproduct of regular processing alone. This may be easily accomplished by porogen incorporation or foaming of the precursor suspension, since the suspensions can be made viscous enough to support large enough bubbles and the air-liquid interface is stable enough to allow for large interfacial area. On the other hand, without modification the pores are sufficiently large to facilitate cell migration and survival in the center of a three-dimensional scaffold of high WPI concentration. Modification of the process to produce higher porosity should also improve both throat diameters and interconnectivity to acceptable levels for the application. In aspects excepting pore network structure, WPI has been shown to possess many favorable characteristics that would lead to its success as a bone regeneration scaffold. At high concentrations (as in 35WPI and 45WPI) it forms a hard gel [16]. Its ability to support the proliferation of osteoblast-like MC3T3-E1 cells in two-dimensional culture for a period of weeks has been established, as well as its ability to enable their mineralization [17]. Under processing conditions as those described here, WPI films have shown a lack of immunogenic response up to 60 days post-implantation, and suitable biodegradability [31]. Once the fabrication technique is refined to address the shortcomings of the overall porosity and interconnectivity, great strides will be made towards the feasible implementation of WPI as a bone regeneration scaffold.
The unique analysis used in this study allowed for an unambiguous evaluation of the material in question based on the known design criteria for pore network structures. The low interconnectivity and gradients in the material served to illustrate the importance of distinguishing the spatial variations within a solid. The value of this information is already understood and has been applied as a means of quantifying the degree of bone ingrowth during \textit{in vivo} scaffold testing \cite{27}, for modeling permeability based on pressure gradients through titanium foams \cite{32}, and for hydroxyapatite density distributions of printed scaffolds \cite{33}. Such information has also been employed outside the field, e.g., in the characterization of packed beds \cite{34-35}. The same techniques can be applied to scaffold characterization, pre-implantation, as has been demonstrated here.

\textbf{6.5. Conclusions}

Microcomputed tomography coupled with pore network modeling was successfully used to gather a wealth of information concerning scaffolds containing both interconnected and isolated pores. The analysis revealed relationships of the scaffold pore network characteristics to the WPI concentration of the hydrogels. The porosity showed a strong dependence on concentration, though pore size distribution and throat size were roughly independent above a certain threshold WPI concentration. The tested WPI scaffolds were fabricated relying solely on entrapped air as a pore-forming agent during agitation, and based on the results, this method is insufficient to create the porosity or interconnectivity required for tissue regeneration. Active incorporation through such techniques as foaming or porogen use will be necessary. It was, however, discovered that a WPI gel precursor suspension can lend itself to such a process for two reasons. First, the suspension viscosity was found to increase roughly exponentially with protein concentration, so high viscosities may be reached, which will in turn result in strong drag forces to entrap rising
bubbles during thermal gelation. Second, whey protein prefers the air-liquid interface, and will serve to stabilize the increased surface area created by gas foaming.

The spatial distributions of the pore network properties especially highlighted the advantages of XMCT analysis. It was found for the WPI scaffolds that porosity and pore size varied with radial position, and further that the shape of the trend was WPI-concentration dependent. The throat diameters and pore coordination numbers were uniform throughout the porous scaffolds. Access to this information can be used to design gradients into tissue scaffolds and quantitatively evaluate them, or for quality assessment and regulation of medical device compliance.

6.6. References


Chapter 7: Method for Electrospinning Pure Whey Protein Isolate and Characterization of Electrospun Films

7.1. Introduction

The ability of nanofibrous polymer scaffolds to mimic the extracellular matrix of tissue has been repeatedly demonstrated and its implications to the field of tissue engineering are under considerable study [1-3]. The micro- and nano-structure of biomaterials control the bulk physical properties as well as cellular behavior. Creating such structures will achieve the goal of providing a scaffold system with the ability to safely interact and degrade biologically. With the ability to control the scaffold properties on the micro- and nano-scale, scaffolds may be better designed to elicit effective tissue growth and repair.

By the electrospinning process, continuous micro- and nano-fibrous structures are created rapidly and inexpensively. In this process, a voltage is applied to a needle, creating an electric field between polymer solution flowing through the needle and a grounded collection surface some distance below. As voltage increases, the charge difference created between the solution and the plate becomes stronger and the round droplet exiting the needle is drawn out to form a cone, known as the Taylor cone [4]. Once a critical voltage is reached, a fiber jet is ejected from the tip of the Taylor cone. As the jet travels from the tip of the cone, the solvent evaporates leaving behind a charged fiber that continues to make its way to the oppositely charged plate depositing itself as one long, ultrafine strand (Fig. 7.1).

Certain material and process variables such as polymer type, concentration, applied voltage, solution flow rate, collector distance, needle inner diameter, etc. have been found to have a
distinct effect on the nanofibrous structure produced and its properties [5-6]. These variables may be tuned in order to produce desired characteristics of the nanostructure by altering the uniformity, surface area per volume, porosity and pore size, absorbance capacity, smoothness, etc. [1, 5-6]. With control over these characteristics, electrospun nanofibers have great potential for application in the filtration, coatings, and textile industries [7-9]. The medical industry also stands to benefit greatly from the application of nanostructures with applications serving to advance areas such as wound healing, biosensors, drug delivery, and particularly tissue engineering [10-13].

The purpose of this study was to describe a method of electrospinning whey protein isolate (WPI) – a mixture of milk proteins – and characterizing the non-woven mats created by the process and their response to material and process variables. WPI gels in bulk form have been demonstrated to promote the proliferation and differentiation of osteoblasts in vitro as described previously in detail [14]. By electrospinning the material, structures of high porosity and interconnectivity can be constructed to further approach the ideal set of...
properties for a bone tissue regeneration scaffold. Additionally, electrospun WPI and its components hold considerable promise for the applications of in vitro culture surface, biosensing, wound healing, and drug delivery being considered by the research in the field of nanotechnology.

7.2. Materials and Methods

7.2.1. Materials

Whey protein isolate (WPI) powder (Davisco Foods International, Eden Prairie, MN) containing 97.6% protein by weight was used as provided. Trifluoroacetic acid (TFA) (>98%) was purchased from Sigma-Aldrich (St. Louis, MO).

7.2.2. Electrospinning

Solutions containing 0.30-0.60 g WPI/mL TFA were prepared in a well-vented fume hood by gradual addition of WPI to TFA and vortexed to mix in a 50 mL centrifuge tube. Each solution was loaded into a 5 mL polypropylene lock-tip syringe and fed through Tygon® R 3603 laboratory tubing, and out of a 38.1 mm (1.5 in), 18-gauge blunt-ended needle using a KDS 100 syringe pump (kd Scientific Inc., Holliston, MA). Electrospinning was performed in a custom apparatus constructed in-house. The needle was connected to a high-voltage source and placed in an adjustable, insulating stand orthogonally to a grounded copper collection plate. The needle and plate construct was encased in an insulated polycarbonate chamber.

Operation variables were manipulated to determine the operational range and impact on the electrospun fibers. Flow rate was varied from 0.1 to 2.5 mL per hour; applied voltage was varied from 10 to 30 kV; the distance between the needle and the collection plate was varied from 120 to 180 mm. Electrospun fibers were collected with forceps and stored in nitrogen in
a sealed centrifuge tube covered in aluminum foil until characterized, to protect against changes due to light or oxygen.

### 7.2.3. Fiber Characterization

The electrospun fibers were sputter-coated with gold and viewed with a Cambridge Stereoscan 260 scanning electron microscope (SEM) at 4 kV. Micrographs of the fibers were used to compare fiber diameter, uniformity, and general appearance and to gauge the dependence of these properties on process variables. Image analysis for fiber diameters was performed using ImageJ for Microscopy software.

### 7.3. Results

WPI solutions in TFA were able to be electrospun under a range of conditions, yielding various morphologies of micro- and nano-fibers (Fig. 7.2).

#### 7.3.1. Concentration Effects

Since samples initially displayed good spinning characteristics at a voltage of 18.6 kV, a flow rate of 0.1 mL/hr, and a collector distance of 12 cm, these control parameters were used when varying concentration. Protein content resulting in spinnable solutions ranged from 0.40 g WPI to 0.55 g WPI per mL TFA. Increasing the protein concentration was found to increase fiber diameter fourfold across the range of spinnable solutions (Fig. 7.3). The

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**Fig. 7.2** – Fiber morphology depends on processing parameters. (a) Beading effect and heterogeneous fibers, (b) thick, uniform fibers in interconnected network, and (c) thin, smooth, uniform fibers. Scale bar = 20 μm.
change in concentration also affected beading in the sample, or the presence of nanobeads among the fully-formed fibers. This undesired phenomenon was observed in lessening degrees between 0.40 and 0.50 g WPI/mL TFA, then to a greater extent at 0.55 g WPI/mL TFA – the boundary of the spinnable range. Solutions of concentration above and below the tested range did not yield electrospun fibers. Outside the range the rheological properties of the spinning solutions were unsuitable for fiber formation. At too-low concentrations of WPI, viscosity was not high enough to form a rapidly elongating fiber. At too-high concentrations, the solution took on non-newtonian behavior and exhibited gelation within the needle in the apparatus.

7.3.2. Flow Rate Effects

Solutions of 0.45 g WPI/mL TFA were subjected to 18.6 kV with a collector distance of 120 mm to test the effects of the solution flow rate on the spinnability of the solution and resulting fiber diameters. Flow rates were varied from 0.1 mL/hr to 2.5 mL/hr. The data suggested a modest rise in fiber diameter between flow rates of 0.1 and 1.0 mL/hr, with roughly constant diameter between 1 and 2.5 mL/hr (Fig. 7.4).

Fig. 7.3 – Dependence of electrospun fiber diameter on protein concentration. Empty markers represent non-spinning solutions. Fig. 7.4 – Dependence of electrospun fiber diameter on solution flow rate.
7.3.3. Voltage Effects

To test the effect of voltage on the electrospun WPI fiber morphology and diameter, solutions of 0.45 g WPI/mL TFA were flowed at 0.5 mL/hr for a distance of 120 mm towards the collection plate. Voltages between 0 and 30 kV were applied to the solutions. The range allowing for successful electrospinning was between 12.5 and 27.5 kV. For most of the spinnable range of voltages, the fiber diameter was proportional to the voltage applied (Fig. 7.5). The exception occurred at the voltage corresponding to the onset of spinnability – 12.5 kV. Scanning electron micrographs highlighting fiber morphology for the tested voltages are shown in Fig. 7.6. The fiber diameters resulting from this voltage were the largest in the range and appeared wavy and discontinuous (Fig. 7.6a). The intermediate voltages were more suitable, forming longer, smooth, straight fibers as shown in Fig. 7.6b-c. Additionally, fiber diameter at 17.5 kV was less than half that for fibers spun at 12.5 kV. Branching of the fibers was observed throughout the range, with the intermediate voltages minimizing branching and resulting in the smoothest, most continuous strands. Overall, nanofibers as narrow as 150 nm were observed, corresponding to an applied voltage of 22.5 kV.

Fig. 7.5 – Dependence of electrospun fiber diameter on applied voltage. Empty marker indicates non-spinning voltage

Fig. 7.6 – WPI fibers electrospun at (a) 12.5 kV, (b) 17.5 kV, (c) 20.0 kV, (d) 22.5 kV, and (e) 27.5 kV.
7.3.4. Collector Distance Effects

The effect of the vertical distance traversed by the fibers to reach the collection plate was tested by flowing WPI solutions of 0.45 g WPI/mL TFA at a rate of 0.5 mL/hr and applying 18.6 kV to the needle. The distance from the tip of the needle to the collection plate was varied from 120 to 200 mm. Electrospun fibers formed for distances between 120 and 180 mm. Fiber diameter was a strong function of spinning distance; as the distance to the collection plate increased, fiber diameter increased (Fig. 7.6), but so did fiber branching as the smoothness of the fibers decreased.

Variations in ambient temperature and humidity are known to impact the properties of electrospun fibers [15]. These were not controlled in this study and may have influenced the results observed.

7.4. Discussion

The effects of material and processing parameters on the properties of electrospun fibers have been studied previously at considerable length, and the phenomenon causing each observed effect is understood. In a study by Tan et al., it was determined that most of the parameter effects may be attributed to one of two classes of variables dictating the properties. The first is the mass of the polymer or protein. As the mass of the polymer being spun increases, so does fiber diameter, until outside the range of spinnability. Protein concentration (as well as molecular weight), flow rate, and applied voltage all effectively change the mass of the protein being
spun. Thus increasing each of these properties should raise the fiber diameter within the applicable range. The second class of variables is the electrical force causing jet elongation. As the electrical force increases it creates a greater drive for jet elongation and the fiber diameter decreases. Applied voltage as well as solvent conductivity influence the electrical force to which the fluid is subjected. Thus an increase in either decreases the fiber diameter. Notably, applied voltage brings about competing effects, resulting in a roughly constant fiber diameter for the intermediate range of voltages [5].

The variable of distance from the needle to the collector plate acts in two ways. First, increasing the distance weakens the electrical field generated by the voltage drop, resulting in larger diameters. Second, increasing the distance increases the flight time of the fibers, allowing the solvent more time to evaporate, changing the morphology of the fiber [16].

This work presents the first instance of WPI electrospinning. Pure WPI solution was successfully electrospun under a range of conditions and the fibers were characterized for optimum properties based on the material and process variables. Although the qualitative trends and effects for polymer and protein solutions during electrospinning are known, it was important to determine the applicable spinning ranges of parameters and to quantify the specific effects they had on the fiber diameter for the system under study. The trends found in this work showed agreement with observations made for other polymers, and were consistent with the theory driving them. Raising protein concentration or flow rate increased fiber concentration, while raising applied voltage showed a minimal increase for the range of continuous, non-beading fibers. Increasing the collector distance from the needle substantially increased the fiber diameter, up to the threshold where the electric field was too weak to generate fibers.
7.5. Conclusions

A successful method of electrospinning never-before spun WPI has been established. WPI was fully dissolved in trifluoroacetic acid and electrospun into non-woven mats of fibers. The operating range for fiber formation was established, and fibers ranging in diameter from 150 to 4800 nm were created. This technique may hold implications towards bone tissue engineering and other novel applications of WPI.

7.6. References


Chapter 8: Conclusions, Optimization, and Future Outlook

8.1. Conclusions

The purpose of the project was to design a material based on a high-concentration WPI gel, and optimize its properties by tuning material and process variables. In order to perform such an optimization, a thorough characterization of a previously untested material had to be performed to evaluate the contribution of each variable to the various material properties and cell-material interactions. Some of the most important features of a bone regeneration scaffold were highlighted and selected as top priority for characterization. These were (1) the mechanical properties, namely, the compressive strength and modulus of the naïve gel; (2) the hydration behavior of the naïve gel; (3) the mechanical properties of reinforced gel composites; (4) biocompatibility and cell-material interactions; (5) pore network structure of the naïve gel and (6) morphology of electrospun WPI fibers.

8.1.1. Mechanical Properties of the Naïve Gel

The compressive properties of WPI gels depended on their compositions. Compressive strength was highest for a gel containing 35% w/v WPI and 2.5-10 mM CaCl₂. Elastic modulus was proportional to WPI concentration, but was highest between 5 and 15 mM CaCl₂. These trends corresponded to the aggregate size and the size of interconnects between aggregates that formed the gel. The most stable network corresponded to the gel with the highest mechanical strength.

8.1.2. Hydrogel Swelling Properties

The composition of the gels dictated both their initial water content and their swelling properties. Initial water content of the gels was in good agreement with a mass balance that could be
performed on each gel sample from its fabrication parameters. The swelling was dependent on WPI concentration and to a greater extend, CaCl₂ concentration. Gels of higher WPI content or higher CaCl₂ content took up more water mass and grew to larger dimensions than gels of lower WPI or CaCl₂ content. The difference between concentrations was large for low-WPI or low-CaCl₂ gels, but became roughly constant between high-WPI or high-CaCl₂ concentration gels. The swelling is primarily driven by the charge density gradient between the inside of the gel and the diffusing solvent outside the gel. Both WPI and CaCl₂ contain added charges that increase the driving force for swelling. The plateaus in the trend were attributed to the increased crosslinking density and internal charge neutralization, restricting the swelling. Throughout the range of compositions, mass change was greater than volume change upon swelling. The disparity can be associated with the added macro- and nanoporosity characteristic of higher WPI and CaCl₂ gels, respectively. The filling of macro- or nanopores does not bring about a significant volume change, though the mass of the water is still increasing.

**8.1.3. Mechanical Properties of Reinforced Composites**

Polysaccharide additives were incorporated into WPI precursor suspensions for gelation of conventional composites. Five polysaccharides were tested. Of these, amylose, cellulose, and dextran detracted from the mechanical properties of the gel. Chitosan showed possible increase in modulus but a decrease in compressive strength over the naïve gels. The behavior was probably caused by the presence of primary amines in the backbone, serving to increase interfacial forces between phases. Amylopectin caused an increase in strength as well as modulus over those of naïve gels across the range of WPI concentrations. The best properties were achieved for 40% w/v WPI, 10 mM CaCl₂, and 0.2 g amylopectin per g WPI, but they still fell short of the required range of mechanical properties. The best composite showed 70% of the
necessary strength, but still one order of magnitude below the necessary compressive modulus. It is likely, however, that these values may still be reached by shifting to a nanocomposite, with a filler on the nanoscale, functionalized with side groups that will strongly interact with the amino acid side groups of the protein.

8.1.4. Biocompatibility and Cell-Material Interactions

In the two-dimensional in vitro experiments performed, the cells showed a high affinity towards the naïve WPI gels as well as the composites containing amylopectin. The cells adhered with high efficiency by static seeding in short seeding times (1 hour), with enough force to withstand subsequent rinsing prior to incubation. After short incubation they displayed the desired flat, stellate morphology indicating high-quality adhesion to the surface (See Appendix E).

The cells were found to remain viable for long-term incubation (3 and 4 weeks in culture). On all tested gel surfaces – spanning the feasible range of compositions – the cells exhibited exponential growth kinetics until saturation of the scaffold occurred. Generally, the cell density data were well-described by an exponential growth model for growth up to 14 days, and by the more complex Gompertz function for growth up to 21 days, where deceleration of growth caused by scaffold saturation took place.

The proliferation kinetics depended to a degree on scaffold compositions. Seeding efficiency was enhanced by scaffold concentrations of at least 35% w/v WPI and 0-10 mM CaCl₂. The growth rate constants were roughly independent of composition, with a suggested increased rate for increasing CaCl₂ and amylopectin concentration. The rate of retardation constant did not exhibit a compositional dependence.

When provided with ascorbic acid to induce differentiation and an inorganic phosphate source, the cells were able to form a mineralized extracellular matrix during a 28-day culture period.
Calcium and phosphorous were detected on all acellular scaffolds initially containing calcium in the matrix, indicating the deposition of a calcium phosphate layer on the surface. Saturation of almost all seeded scaffolds was reached during the 28-day period, and scaffolds seeded with the differentiating subclone of the cell line showed mineralized ECM, though only sporadic mineralization was seen on surfaces containing 0 mM CaCl\(_2\). This indicated that the calcium source built into the matrix aided in scaffold mineralization. Scaffolds seeded with non-mineralizing subclones served as negative controls and showed no evidence of calcium phosphate formation, though the cultures thrived on the WPI and composite surfaces.

The proliferation and mineralization behavior of the WPI scaffolds were found to be suitable for use in bone regeneration. All tested scaffolds supported both proliferation and mineralization (and implicitly, osteoblastic differentiation) of progenitor cells. The optimal cellular behavior was observed for scaffolds contained high WPI, low-to-medium CaCl\(_2\), and high amylopectin concentrations.

**8.1.5. Pore Network Structure of Naïve Gels**

The WPI gel pore network structure was found to depend heavily on WPI concentration in the gel precursor suspension. It was determined that a threshold WPI concentration was necessary to obtain any detectable pore content in the material. This threshold is between 20% and 35% w/v WPI, and is dictated primarily by the viscosity of the gel precursor suspension. Between 20% and 35% w/v WPI, the suspension undergoes a shift from Newtonian to shear-thinning characteristics, and experiences a 33-fold increase in viscosity. The added suspension viscosity imparts a drag force great enough to trap large air bubbles introduced during suspension preparation that escape during gelation when the viscosity is lower.
The pore size distributions for the porous gels (35% and 45% w/v WPI) were within the proper range for successful 3D cell culture. Using the current processing technique, the highest porosity attained was 17.8%, which is far below the required range for bone regeneration. Pore interconnectivity and inter-pore throat diameters were also found to be inappropriately low for the application. Fortunately, additional void volume can be readily incorporated by modifying the current scaffold fabrication technique, as discussed in Chapter 7 and in Section 8.3.1.

8.1.6. WPI Electrospinning

The preliminary work in the electrospinning of WPI was presented as a possible solution to the pore network structure limitations encountered in Chapter 6. A novel method for electrospinning pure WPI was presented, and the operational range of variables was established. The operational parameters leading to the optimal fiber morphology was determined and recommended for use in future experiments in the electrospinning of WPI. The greatest hurdles in the technique include the water solubility of the fibers following electrospinning and the presence of trace solvent in the mat. Both concerns are addressed in Section 8.3.1, and further experimental work is required to enable the use of this method of scaffold fabrication.

8.2. Scaffold Optimization

Following these results, the optimal scaffold formulation recommended was 40% w/v WPI, 10 mM CaCl₂, and 0.2 g amylopectin per g WPI in water. This composition is optimal for thermal gelation at 80°C for approximately 12 minutes for every millimeter of characteristic length. At this composition, the compressive strength and compressive modulus were the highest achieved, the porosity was near its highest, and the pore size distribution was favorable. Seeded cells demonstrated high seeding efficiency (>75% after 1 hour), rapid proliferation, and unobstructed differentiation and mineralization.
This recommendation is based on the combined optimum determined from this work. Modifications must be made to improve the mechanical properties and the porosity and interconnectivity of the scaffold. As the scaffold showing the greatest promise, this optimal formulation should serve as the launch point for future experiments.

8.3. Future Paths

As is common with accelerating topics of research, every question answered in the scope of this project raised several more. It would be advantageous to pursue many of these questions and widen the understanding of the WPI gel material within the context of application toward bone tissue regeneration, as well as investigate its suitability in other novel applications. Some of these avenues of possible development are outlined here.

8.3.1. Attaining the Ideal Scaffold Pore Network

The macroporosity of WPI gels was addressed in Chapters 2, 5, and 7. Several pore-network related properties were found inappropriate for the application, and several ways to address the discrepancies have been raised. The first was to fabricate the scaffolds using electrospinning, and the groundwork was laid in Chapter 7. However, the current technique is far from ideal. The technique requires the use of trifluoroacetic acid (TFA) – a toxic solvent – the removal of which must be exhaustive in order to enable spun scaffolds to come into contact with a biological environment. Either the development of a purification protocol or a technique to spin WPI in water or another benign solvent would be required. Additionally, in the form presented in Chapter 7, the electrospun fibers are highly soluble in water as well as ethanol – a critical obstacle for use in an aqueous environment. It is believed that the fiber network can be cross-linked and rendered insoluble as seen for bovine serum albumin (BSA), which is the most water-soluble component of WPI. Aged BSA fibers have been found to lose their solubility [1]. It is
likely that the same can be achieved for WPI fibers and perhaps accelerated using a strong UV source. This possibility should be investigated to solve the solubility problem of electrospun WPI. It must be noted that if the challenges of electrospinning WPI can be addressed, it will still be necessary to combine it with a technique to incorporate macroporosity into the scaffold, since electrospun non-woven mats generally contain nanopores, but no pores on the order of hundreds of micrometers, as those necessary for a bone tissue scaffold.

A second possible method for increasing the overall porosity and interconnectivity of WPI scaffolds is a technique known as salt leaching. Many cases in the literature can be found where NaCl crystals of a specific size range were incorporated into a non-solvating, organic solution of the matrix polymer and served as place-holders for matrix pores. The salt was later dissolved with water, leaving behind an interconnected, high-void-volume network [2-4]. Although NaCl cannot serve this function in an aqueous precursor suspension as the one in this project, a similar procedure may be employed if optimized for the system. Sodium sulfate is almost water-insoluble at room temperature, but solubility increases by an order of magnitude at 32.4ºC [5]. This phenomenon may be employed to build porosity into WPI gels by incorporating microparticles of Na₂SO₄ into the precursor suspension, then dissolving the salt at 32.4ºC. By characterizing the contribution of the salt particles and refining the process to yield the proper porosity, interconnectivity, and pore size, the procedure may be optimized to overcome the pore network limitations.

A third possible method to correct the observed pore network is a variation of gas foaming during gelation. Preliminary studies have shown that incorporation of ethanol and a small concentration of surfactant, such as Triton-X100, elicits a rapid foaming response during gelation at 80ºC, due to the boiling of ethanol in the system. If the technique can be characterized and
optimized to predictably achieve the proper porosity and pore size by controlling composition, the method can serve to overcome the current limitations.

### 8.3.2. Nanocomposite Design and Testing

At the conclusion of this project, the mechanical properties of the strongest composite formed and presented in Chapter 4 were still insufficient for load-bearing applications of the material. It would be useful to investigate the contribution of a nano-phase, crystalline, polysaccharide filler. If other material combinations are an indication, the use of nano-crystalline cellulose or amylopectin could drastically improve the composite mechanical properties, launching them into the range appropriate for withstanding normal stresses. Different fillers should be tested, as well as functionalization of the nanophase. Polysaccharide nanocrystals can be functionalized with primary amines or carboxylic acids, or even some sulfhydryl groups that would contribute to strong interfacial forces binding filler to protein matrix.

### 8.3.3. Degradation Studies

The success of an implantable bone regeneration scaffold hinges on its degradation properties and mechanism. The scaffold should clear the site of injury within a time period on the order of months. The degradation should not cause the material to lose load-bearing capacity prior to the formation of enough regenerating bone to take over the function. Also, the degradation products must be non-toxic, and should not accumulate in any organ. The degradation properties of WPI gels were not characterized in this work, but should be addressed in the future as an important aspect of scaffold design.

### 8.3.4. 3D Scaffold Testing

All cellular response experiments (Chapter 1, 6) in this project were conducted in two dimensions – on a flat surface. Bone and the cells that comprise it have inherent three-dimensional characteristics, such as intercellular sensing, environmental sensing and response,
and specific surface area considerations. Having established WPI gel as a viable growth surface, the next step is to evaluate its behavior in three-dimensional culture.

### 8.3.5. Stem Cell Response

*In vitro* studies throughout this project used a model immortal cell line of osteoblast-like cells. The choice of cell line was common, and universally accepted as a useful preliminary indication of cell behavior. These tests should be followed up with similar tests using stem cells as well as a suitable primary cell line. These are the cells that will be responsible for osteogenesis in the implant environment, so their response to WPI gels should be extensively characterized.

### 8.3.6. Immunogenicity Studies of WPI

Concerns have been raised in response to this work regarding the immunogenicity of WPI, barring its use *in vivo*. In this project, WPI was found to be non-cytotoxic, and promoted the adhesion and long-term proliferation of osteoblast cells. This behavior, however, is independent of the possible response in the presence of cells controlling the immune response. A study has been done with WPI-PEG mixed gels implanted subcutaneously in mice, showing that immunogenicity is not a concern for up to 60 days [6]. This greatly supports the assertion that current processing methods (similar to the ones in the cited study) remove immunogenic recognition factors from the network, but a similar study should be conducted to provide irrefutable proof of the material biocompatibility.

### 8.3.7. *In Vivo* Testing

The suggestion to perform a set of *in vivo* experiments goes hand-in-hand with immunogenicity testing, which could be addressed concurrently. Because the implant environment can only be reproduced to a degree *in vitro*, *in vivo* testing cannot be avoided. Bone formation with or without preseeding and preculturing of cells in WPI gels at different lengths of time needs to be measured in order to argue the suitability of WPI scaffolds with confidence.
8.3.8. Other Applications for WPI

The properties of WPI gels were found to vary widely with composition and processing. This versatility can be made useful for applications other than the one explored here. It could be advantageous to determine the potential of WPI for use as wound-dressing, regeneration scaffold of other tissues, such as skin or connective tissue, in electrospun non-woven mats, for controlled release of small molecules, as a chemical sensor or biosensor, or as an environmentally benign, economically favorable column packing material. WPI possesses many attractive characteristics, and its potential is unlikely to be exhausted in the proposed application of bone tissue regeneration.

8.4. References


Appendix A: Cure-Time Profiles for Whey Protein Isolate Gels

The effect of cure time on the mechanical properties of WPI gels was investigated so as to select the cure time leading to the most favorable properties. The relationship was determined by varying the cure times of gels of a representative composition. Compression samples were fabricated as described in Section 2.2.2. For the geometry tested (D = 10 mm, L = 5 cm), the cure time selected was 60 minutes, since it was the shortest time to yield the best properties (Fig. A.1). The results can be translated qualitatively to other gel geometries. The optimal cure time depends on the characteristic length of the gel. In a cylindrical sample of aspect ratio greater than one, as in this experiment, the characteristic length is the radius. A rule-of-thumb that can be used is 12 minutes of cure time per millimeter of characteristic length. This value would correspond to the minimum cure time to yield optimal properties. The results shown in Fig. A.1 dictated the minimum cure times selected for sample fabrication throughout the project.

Fig. A.1 – Mechanical properties of WPI gels as a function of cure time. Plots showing (a) compressive strength, (b) compressive modulus, and (c) break strain as functions of cure time. The results led to the selection of 60 minutes as the optimal cure time for compression samples of WPI gels (radius = 5 mm).
Appendix B: Scanning Electron Micrographs of MC3T3-E1 Cells Adhered to a Whey Protein Isolate Surface

The following micrographs (Figs. B.1-B.25) are provided as an expansion on Fig. 2.8 in Chapter 2. Additional scaffold locations and magnifications were employed to probe several regions of interest. The characterized surface is composed of 45% w/v WPI and 10 mM CaCl₂. The samples were processed as described in Section 2.2.5. The figures are presented in sequences of increasing magnification to maintain the context of highly-magnified images.

Fig. B.1 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.2 – SEM micrograph depicting cells adhered to WPI surface.
Fig. B.3 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.4 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.5 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.6 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.7 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.8 – SEM micrograph depicting cells adhered to WPI surface.
Fig. B.9 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.10 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.11 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.12 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.13 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.14 – SEM micrograph depicting cells adhered to WPI surface.
Fig. B.15 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.16 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.17 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.18 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.19 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.20 – SEM micrograph depicting cells adhered to WPI surface.
Fig. B.21 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.22 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.23 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.24 – SEM micrograph depicting cells adhered to WPI surface.

Fig. B.25 – SEM micrograph depicting cells adhered to WPI surface.
Appendix C: Detailed Results for Swelling Studies to Supplement Chapter 3

C.1. Individual-Gel Swelling Curves

The swelling properties of WPI gels were discussed in Chapter 3, and swelling curves were presented for gels of varying compositions. For the sake of concision and ease of comparison, multiple curves were shown in every plot. For the sake of clarity, error bars were omitted. The following figures (Figs. C.1-C.12) present the swelling data of each composition individually, with the standard deviation of each datum included.

C.2. SAS Regression and Output

SAS statistical software was used to perform the power-law regressions in the Chapter 3 analysis. Only part of the regression output was used in the chapter, so a copy of sample program code is shown in Fig. C.13 and direct reproductions of all output files are provided in Sections C.2.1-C.2.4.

Fig. C.1 – Swelling curves for gels composed of 20% w/v WPI and 10 mM CaCl₂. (a) Swelling defined by mass increase; (b) swelling defined by volume increase.
Fig. C.2 – Swelling curves for gels composed of 25% w/v WPI and 10 mM CaCl₂. (a) Swelling defined by mass increase; (b) swelling defined by volume increase.

Fig. C.3 – Swelling curves for gels composed of 30% w/v WPI and 10 mM CaCl₂. (a) Swelling defined by mass increase; (b) swelling defined by volume increase.
Fig. C.4 – Swelling curves for gels composed of 35% w/v WPI and 10 mM CaCl₂. (a) Swelling defined by mass increase; (b) swelling defined by volume increase.

Fig. C.5 – Swelling curves for gels composed of 40% w/v WPI and 10 mM CaCl₂. (a) Swelling defined by mass increase; (b) swelling defined by volume increase.
Fig. C.6 – Swelling curves for gels composed of 45% w/v WPI and 10 mM CaCl₂. (a) Swelling defined by mass increase; (b) swelling defined by volume increase.

Fig. C.7 – Swelling curves for gels composed of 35% w/v WPI and 0 mM CaCl₂. (a) Swelling defined by mass increase; (b) swelling defined by volume increase.
Fig. C.8 – Swelling curves for gels composed of 35% w/v WPI and 5 mM CaCl₂. (a) Swelling defined by mass increase; (b) swelling defined by volume increase.

Fig. C.9 – Swelling curves for gels composed of 35% w/v WPI and 10 mM CaCl₂. (a) Swelling defined by mass increase; (b) swelling defined by volume increase.
Fig. C.10 – Swelling curves for gels composed of 35% w/v WPI and 20 mM CaCl$_2$. (a) Swelling defined by mass increase; (b) swelling defined by volume increase.

Fig. C.11 – Swelling curves for gels composed of 35% w/v WPI and 35 mM CaCl$_2$. (a) Swelling defined by mass increase; (b) swelling defined by volume increase.
Fig. C.12 – Swelling curves for gels composed of 35% w/v WPI and 50 mM CaCl₂. (a) Swelling defined by mass increase; (b) swelling defined by volume increase.

```
proc model data=work.WPIVOLUME;
  WPI_1=a*t**b;
  WPI_2=c*t**d;
  WPI_3=f*t**g;
  WPI_4=h*t**j;
  WPI_5=k*t**l;
  WPI_6=m*t**n;
  fit WPI_1 WPI_2 WPI_3 WPI_4 WPI_5 WPI_6 / method=marquardt;
  maximizer=10000 converge=0.000001 cov prl=both;
run;
```

Fig. C.13 – Sample program code for swelling-curve regressions. This code was used for all fits, with data file names and variables as the only changes between runs.
C.2.1. Output for Mass Change with respect to WPI

The SAS System 14:47 Wednesday, March 10, 2010

The MODEL Procedure

Model Summary

Model Variables 6
Parameters 12
Equations 6
Number of Statements 6

Model Variables WPI_1 WPI_2 WPI_3 WPI_4 WPI_5 WPI_6
Parameters a b c d f g h j k l m n
Equations WPI_1 WPI_2 WPI_3 WPI_4 WPI_5 WPI_6

The 6 Equations to Estimate

WPI_1 = F(a, b)
WPI_2 = F(c, d)
WPI_3 = F(f, g)
WPI_4 = F(h, j)
WPI_5 = F(k, l)
WPI_6 = F(m, n)

NOTE: At OLS Iteration 16 CONVERGE=1E-6 Criteria Met.

The SAS System 14:47 Wednesday, March 10, 2010

The MODEL Procedure

OLS Estimation Summary
Data Set Options

DATA= WPIMASS

Minimization Summary

Parameters Estimated  12
Method                Marquardt
Iterations            16
Subiterations         18
Average Subiterations 1.125

Final Convergence Criteria

R                    6.475E-7
PPC(l)               2.592E-7
RPC(l)               0.000016
Object               1.713E-9
Trace(S)             0.00109
Objective Value      0.000818
Lambda               1.801E-6

Observations Processed

Read     9
Solved   9
Used     8
Missing  1

The SAS System         14:47 Wednesday, March 10, 2010

The MODEL Procedure

Nonlinear OLS Summary of Residual Errors
Nonlinear OLS Parameter Estimates

| Parameter | Estimate  | Std Err | t Value | Pr > |t| |
|-----------|-----------|---------|---------|------|---|
| a         | 1.017444  | 0.0125  | 81.52   | <.0001|
| b         | 0.011444  | 0.00273 | 4.19    | 0.0058|
| c         | 1.004711  | 0.00622 | 161.46  | <.0001|
| d         | 0.013438  | 0.00138 | 9.77    | <.0001|
| f         | 0.994168  | 0.00405 | 245.51  | <.0001|
| g         | 0.018511  | 0.000898| 20.60   | <.0001|
| h         | 1.024829  | 0.0121  | 84.52   | <.0001|
| j         | 0.025847  | 0.00258 | 10.01   | <.0001|
| k         | 1.029625  | 0.0145  | 71.24   | <.0001|
| l         | 0.027027  | 0.00306 | 8.83    | 0.0001|
| m         | 1.01197   | 0.00728 | 138.98  | <.0001|
| n         | 0.026003  | 0.00157 | 16.55   | <.0001|

Parameter Wald
95% Confidence Intervals

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<th>Value</th>
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<th>Upper</th>
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The MODEL Procedure

Parameter Likelihood Ratio
95% Confidence Intervals

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Number of Observations

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The SAS System 14:47 Wednesday, March 10, 2010
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### C.2.2. Output for Volume Change with respect to WPI

The SAS System 14:47 Wednesday, March 10, 2010

116

The MODEL Procedure

Model Summary

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<td>Equations</td>
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Model Variables  WPI_1 WPI_2 WPI_3 WPI_4 WPI_5 WPI_6
Parameters  a b c d f g h j k l m n
Equations  WPI_1 WPI_2 WPI_3 WPI_4 WPI_5 WPI_6

The 6 Equations to Estimate

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\begin{align*}
 WPI_1 &= F(a, b) \\
 WPI_2 &= F(c, d) \\
 WPI_3 &= F(f, g) \\
 WPI_4 &= F(h, j) \\
 WPI_5 &= F(k, l) \\
 WPI_6 &= F(m, n)
\end{align*}

NOTE: At OLS Iteration 16 CONVERGE=1E-6 Criteria Met.

The MODEL Procedure
OLS Estimation Summary

Data Set Options

DATA= WPIVOLUME

Minimization Summary

Parameters Estimated \quad 12 \\
Method \quad \text{Marquardt} \\
Iterations \quad 16 \\
Subiterations \quad 20 \\
Average Subiterations \quad 1.25

Final Convergence Criteria

\begin{align*}
 R &= 2.979E-7 \\
 \text{PPC}(n) &= 2.055E-7 \\
 \text{RPC}(n) &= 0.000031 \\
 \text{Object} &= 2.601E-9 \\
 \text{Trace}(S) &= 0.000619
\end{align*}
### Nonlinear OLS Summary of Residual Errors

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</table>

### Nonlinear OLS Parameter Estimates

<p>| Parameter | Estimate | Std Err | t Value | Pr &gt; |t| |
|-----------|----------|---------|---------|------|---|
| a         | 0.993443 | 0.0118  | 84.12   | &lt;.0001 |
| b         | 0.011003 | 0.00265 | 4.15    | 0.0060 |
| c         | 0.996612 | 0.00537 | 185.58  | &lt;.0001 |
| d         | 0.008595 | 0.00121 | 7.13    | 0.0004 |
| f         | 1.001212 | 0.000181| 123.42  | &lt;.0001 |
| g         | 0.011126 | 0.00181 | 6.16    | 0.0008 |</p>
<table>
<thead>
<tr>
<th>Parameter</th>
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<th>Upper</th>
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The MODEL Procedure

Parameter Likelihood Ratio

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<th>Upper</th>
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The SAS System 14:47 Wednesday, March 10, 2010

Parameter Likelihood Ratio

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<th>Upper</th>
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\[
\begin{array}{cccc}
\text{f} & 1.0012 & \cdot & 2.7755 \\
g & 0.0111 & -0.3568 & 0.4682 \\
h & 0.9982 & \cdot & 2.7716 \\
j & 0.0127 & -0.3537 & 0.4684 \\
k & 1.0024 & \cdot & 2.7730 \\
l & 0.0135 & -0.3485 & 0.4642 \\
m & 0.9937 & \cdot & 2.7708 \\
n & 0.0131 & -0.3559 & 0.4744 \\
\end{array}
\]

Number of Observations  Statistics for System

Used  8  Objective  0.000464
Missing  0  Objective*N  0.003715

The SAS System  14:47 Wednesday, March 10, 2010

The MODEL Procedure

Covariance of Residuals

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<tr>
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<th>WPI_1</th>
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<th>WPI_3</th>
<th>WPI_4</th>
<th>WPI_5</th>
<th>WPI_6</th>
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Covariances of Parameter Estimates

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<th>b</th>
<th>c</th>
<th>d</th>
<th>f</th>
<th>g</th>
</tr>
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<tbody>
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</table>
C.2.3. Output for Mass Change with respect to CaCl$_2$

The SAS System 16:50 Wednesday, March 17, 2010 1

The MODEL Procedure

Model Summary
Model Variables  
Parameters  
Equations  
Number of Statements  

Model Variables  
Parameters  
Equations  

The 6 Equations to Estimate

CACL2_1 = F(a, b)  
CACL2_2 = F(c, d)  
CACL2_3 = F(f, g)  
CACL2_4 = F(h, j)  
CACL2_5 = F(k, l)  
CACL2_6 = F(m, n)  

NOTE: At OLS Iteration 17 CONVERGE=1E-6 Criteria Met.

The SAS System  16:50 Wednesday, March 17, 2010  2
Average Subiterations     1.058824

Final Convergence Criteria

R                   2.21E-8
PPC(j)             1.142E-8
RPC(j)              5.56E-7
Object             1.18E-12
Trace(S)            0.003171
Objective Value    0.002378
Lambda             1.801E-7

Observations Processed

Read     8
Solved    8

The SAS System         16:50 Wednesday, March 17, 2010   3

The MODEL Procedure

Nonlinear OLS Summary of Residual Errors

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<tr>
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<th>DF Model</th>
<th>DF Error</th>
<th>SSE</th>
<th>MSE</th>
<th>Root MSE</th>
<th>R-Square</th>
<th>Adj R-Sq</th>
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<tbody>
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Nonlinear OLS Parameter Estimates

| Parameter | Estimate | Std Err | t Value | Pr > |t| |
|-----------|----------|---------|---------|-------|---|

150
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<th>Upper</th>
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Number of Observations: 8
Statistics for System Used:
- Objective: 0.002378
- Objective*N: 0.0190

The SAS System 16:50 Wednesday, March 17, 2010

The MODEL Procedure

Covariance of Residuals

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<tr>
<th></th>
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<th>CACL2_4</th>
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Covariances of Parameter Estimates

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### Covariances of Parameter Estimates

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<tr>
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<td>0.0000000</td>
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</table>

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**C.2.4. Output for Volume Change with respect to CaCl₂**

The SAS System 16:50 Wednesday, March 17, 2010 6

The MODEL Procedure

Model Summary
Model Variables          6
Parameters              12
Equations                6
Number of Statements     6

Model Variables  CACL2_1  CACL2_2  CACL2_3  CACL2_4  CACL2_5  CACL2_6
Parameters  a  b  c  d  f  g  h  j  k  l  m  n
Equations  CACL2_1  CACL2_2  CACL2_3  CACL2_4  CACL2_5  CACL2_6

The 6 Equations to Estimate

\[
\begin{align*}
    CACL2_1 &= F(a, b) \\
    CACL2_2 &= F(c, d) \\
    CACL2_3 &= F(f, g) \\
    CACL2_4 &= F(h, j) \\
    CACL2_5 &= F(k, l) \\
    CACL2_6 &= F(m, n)
\end{align*}
\]

NOTE: At OLS Iteration 16 CONVERGE=1E-6 Criteria Met.
Final Convergence Criteria

- $R = 1.482 \times 10^{-7}$
- PPC(b) = $1.503 \times 10^{-7}$
- RPC(b) = 0.000028
- Object = $1.064 \times 10^{-9}$
- Trace(S) = 0.00072
- Objective Value = 0.00054
- Lambda = 0.000115

Observations Processed

- Read 8
- Solved 8

The SAS System 16:50 Wednesday, March 17, 2010 8

The MODEL Procedure

Nonlinear OLS Summary of Residual Errors

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155
Parameter Wald
95% Confidence Intervals

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The SAS System 16:50 Wednesday, March 17, 2010 9

The MODEL Procedure

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The SAS System         16:50 Wednesday, March 17, 2010  10
  
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Covariances of Parameter Estimates

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Appendix D: Supporting Material for Proliferation and Mineralization of MC3T3-E1 Cells on Whey Protein Isolate Scaffolds

Fig. D.1 – Representative scaffold surface used in direct cell count. Every scaffold (shown: 40% WPI; 10 mM CaCl₂; 0% amylopectin), after a prescribed incubation, was fixed, permeablistimized, and stained with Hoechst 33342 nuclear dye. In this representative image, the cell nuclei are seen as bright blue spots. Fourteen such images were captured per repeat, and at least three repeats were used per data point.

Fig. D.2 – Exponential growth curves for CaCl₂ concentrations unsuitable for Gompertz function regression. Proliferation data for MC3T3-E1, subclone 4 cells cultured for 1-21 days on scaffolds containing 35% w/v WPI and 0% amylopectin and 5 or 15 mM CaCl₂. Data points represent averages of at least 3 replications; curves represent regressed fits of the data to the exponential growth model. As a result of the regression: SE = 1.48; k = 0.152 for 5 mM CaCl₂, SE = 0.787; k = 0.190.
Fig. D.3 – SEM micrograph and elemental analysis of mineralized scaffold containing 20 mM CaCl$_2$. The scaffold represented in the micrograph contains 35% w/v WPI and 20 mM CaCl$_2$, and was cultured with MC3T3-E1, subclone 4 (differentiating) cells for 28 days in mineralization medium. Scale bar = 20 μm. EDS spectrum corresponds to a point scan on the background surface of the scaffold. The spectrum contains proof of mineralized content on the scaffold surface.

```sas
proc model data=work.wpidat_g;
   WPI5=a*exp(b*(1-exp(d*t/b)));
   PARMS=(a=100 and b=-10 and d=2);
   fit WPI5 / Method=marquardt maxiter=1000 converge=0.0001 cov prl=both;
run;
```

Fig. D.4 – Sample SAS program for non-linear regression. A variation of this code was used for all non-linear regressions in Chapter 5 and Figure D.2.
Appendix E: Morphology and Internal Structure of MC3T3-E1 Cells on Whey Protein Isolate Scaffolds

E.1. Background
In order to verify that the cells proliferating on whey protein isolate (WPI) scaffold surfaces were healthy, confocal microscopy images were taken to examine the morphology and cytoskeleton structure of MC3T3-E1 cells. By fluorescently labeling the actin molecules that make up the cytoskeleton, the internal structure of the adhered cells could be visually confirmed. This study was conducted in conjunction with the work performed and presented in Chapter 5. While proliferation kinetics are an important measure of scaffold success, the phenotype and morphologies of the cells are equally important, because they are directly correlated with the affinity of cells to the scaffold.

E.2. Methods
Preosteoblasts were cultured on WPI gels using the methods described in chapter 5. Briefly, WPI suspensions were thermally gelled at 80°C to produce scaffolds, sterilized in 200 proof ethanol, then soaked in PBS to remove traces of ethanol from the scaffolds. MC3T3-E1 cells were statically seeded onto the scaffold surfaces, allowed 1 hour to adhere at 37°C in a humid 5% CO₂ atmosphere. The scaffolds were then rinsed with αMEM, provided with 1 mL of medium each, and incubated for 10 days. Prior to imaging, the scaffolds were gently washed with PBS three times, fixed with 4% paraformaldehyde, the cell membranes permeablized with 0.1% Triton-X100, and each sample rinsed with PBS. The scaffolds were stained with rhodamine-conjugated phalloidin for fluorescent microscopy.
E.3. Results

Representative images of cell cytoskeletons are shown in Fig. E.1. These images confirm the high affinity of the cells to the WPI scaffolds, which should greatly contribute to the success of the material should it be implemented in bone regeneration. The actin filaments (red) form a network of straight, organized lines, which is the expected phenotype for a well-adhered, healthy cell. This is distinguished from a disorganized network of spotted actin (Fig. E.2), which was found infrequently on the scaffold surface.

Fig. E.1 – Phalloidin-stained actin filaments (red) by confocal microscopy. The cytoskeleton of MC3T3-E1 cells adhered to WPI gel scaffolds is labeled to visualize the morphology of the cells growing on (a, b) 20% w/v WPI gels and (c, d) 45% w/v WPI gels.
Fig. E.2 – Example of cell exhibiting a poorly-organized cytoskeleton. The stained actin is delocalized instead of being drawn in tight filaments. This morphology indicates a distressed cell. Few such cells were observed on the surfaces of WPI scaffolds.
Appendix F: Supporting Material for Pore Network Characterization of Whey Protein Isolate Gels

F.1. Axial Profiles of Pore Network Characteristics

![Axial profiles of pore network characteristics](image)

Fig. F.1 – Axial profiles of (a) porosity, (b) pore coordination number, (c) pore diameter, and (d) throat diameter for 20% (blue), 35% (red), and 45% (green) w/v WPI gels as obtained by analysis of XMCT data.
F.2. Critical Viscosity Calculation

F.2.1. Theory

The following calculation was performed in order to support a hypothesis concerning the considerable difference in pore size and number observed between the 20% w/v WPI gel and the (more similar) 35% and 45% w/v WPI gels. It is understood that all the pores originated from bubbles in the precursor suspension that remained trapped during curing of the gel. It was also understood that viscosity of the precursor suspension varied widely with WPI concentration. It was therefore hypothesized that the 20WPI precursor solution had too low a viscosity to trap bubbles prior to curing, while the viscosities of the 35WPI and 45WPI suspensions were sufficient to generate a drag force large enough to prevent bubbles from rising to the air-water interface. If the hypothesis is correct, there exists a threshold viscosity that is necessary and sufficient to prevent bubbles of the median diameter (approximately 100 μm) from rising to the interface within the time required for the sample to become solid (approximately 10 minutes). Moreover, the value of the threshold viscosity should lie between the measured viscosity of the 20WPI suspension and that of 35WPI.

F.2.2. Calculation Assumptions

1. Bubbles are in equilibrium: \( \sum F = 0 \)

2. Bubbles are unaffected by one another

3. Density of the WPI suspension is equal to that of water.

4. Bubbles are spheres

5. Cure time, or the time available for bubbles to escape the suspension, is 10 minutes.

6. Velocity pertinent in this system is that which would allow a bubble to traverse the height of the sample within the cure time.
\[ \nu = \frac{\ell}{t} \]

7. Reference bubble size is of the median diameter observed for 35WPI and 45WPI:

\[ D = 100 \mu m \]

**F.2.3. Variable Definitions**

- \( F_B \) = Buoyant force
- \( F_g \) = Gravitational force
- \( F_D \) = Drag force

Index 1 = Fluid 1 in naming convention; Reference to air in the bubble
Index 2 = Fluid 2 in naming convention; Reference to WPI suspension

- \( D \) = Bubble diameter
- \( \nu \) = Velocity of the bubble
- \( \ell \) = Path length, or height of the sample
- \( t \) = Cure time
- \( V \) = Volume

- \( g \) = Gravitational acceleration constant
- \( m \) = mass
- \( \text{Re} \) = Reynolds number
- \( \rho \) = density
- \( \eta \) = dynamic viscosity
- \( \sigma \) = viscosity ratio, \( \eta_2/\eta_1 \)
F.2.4. Force Balance

\[ F_B = F_g + F_D \] (Fig. C.2)

Where

\[ F_B = \rho_2 V_1 g \]

\[ F_g = m_1 g \]

\[ F_D = 6\pi \eta r \frac{v}{D} \left( \frac{1 + \frac{2}{3} \sigma}{1 + \sigma} \right) \]

For translation of a fluid sphere through anotherwise stationary second fluid [1]

F.2.5. Drag Force Expression

Implicit in the above expression for the drag force are a few assumptions. The first is the lack of inertial forces, or the presence of purely viscous forces – as is the case for small objects at low fluid density, low velocity, and high viscosity. These conditions are represented by a low Reynolds number (Re < 0.1) [2]. The Reynolds number varies among our systems of interest, depending on the WPI suspension viscosity. A maximum Reynolds number was calculated, based on the lowest observed viscosity for any suspension.

\[ \text{Re}_{\text{max}} = \frac{\rho_2 v D}{\eta_{\text{min}}} \]

Known quantities are substituted for the variables:
\[ \text{Re}_{\max} = \frac{\rho_s \ell D}{\eta_{\min}} = \frac{(1000 \text{ kg/m}^3)(0.0762 \text{ m})(1 \times 10^{-3} \mu\text{m})}{(600 \text{ s})(0.008 \text{ kg/m} \cdot \text{s})} \]

\[ \text{Re}_{\max} = 1.59 \times 10^{-3} \]

\text{Re}_{\max} \text{ is far below the limit, so the expression for the drag force is valid for the system explored here.}

The second assumption is the spherical shape of the bubbles. This has been proven experimentally for small bubbles subject to surface-active components. The bubbles in our system are small enough to be considered spherical, especially with the interface-favoring WPI present in high concentrations [3].

A third, simplifying assumption is made. Because \( \eta_2 >> \eta_1 \), \( \sigma \) is large – roughly 100. Thus the expression for the drag force simplifies to:

\[ F_D \approx 4\pi \eta r v \]

**F.2.6. Threshold Viscosity from Force Balance**

The known expressions are substituted into the force balance, and the equation is manipulated to solve explicitly for the threshold viscosity, \( \eta_{\text{crit}} \).

\[ \rho_2 V_1 g = m_1 g + 4\pi \eta_{\text{crit}} \frac{D}{2} v \]

\[ \eta_{\text{crit}} = \frac{\rho_2 V_1 g - m_1 g}{4\pi \frac{D}{2} \frac{\ell}{t}} \]

Known values are substituted for variables in the expression:
\[
\eta_{\text{crit}} = \frac{(1000 \text{ kg/m}^3) \left[ \frac{4}{3} \pi \left( \frac{1 \times 10^{-4} \text{ m}}{2} \right)^3 \left( 9.807 \text{ m/s}^2 \right) - \left( 5.245 \times 10^{-13} \text{ kg} \right) \left( 9.807 \text{ m/s}^2 \right) \right]}{4\pi \left( \frac{1 \times 10^{-4} \text{ m}}{2} \right)^3 \left( 0.0762 \text{ m} \right) / 600 \text{ s}}
\]

\[\eta_{\text{crit}} = 0.064 \text{ kg/m·s}\]

This value is eightfold higher than 20WPI viscosity and one order of magnitude lower than 35WPI viscosity – thus supporting the hypothesis that the difference in viscosity led to the onset of porosity.

**F.3. References**


Vita

Mia Dvora earned her Bachelor of Science in chemical engineering with a minor in chemistry from the University of Florida in April, 2005. She continued her studies at Louisiana State University and A&M College where she conducted research in biomaterials and tissue engineering. For two years she participated in the Craft for Macromolecular Creativity IGERT program at the university and in 2009 she received the DOW Chemical Award for Excellence in Macromolecular Studies. Ms. Dvora presented her research at international conferences such as the 2007, 2008, and 2009 annual meetings of the American Institute of Chemical Engineers, the biannual 2008 International Symposium on Polymer Physics, and the 238th meeting of the American Chemical Society in 2009. In 2010 she graduated with a Doctor of Philosophy in chemical engineering and a minor in biological and agricultural engineering from Louisiana State University and A&M College.