

**Roles of Polymer Crosslinking Density and Crystallinity in
Regulating Surface Characteristics and Pre-osteoblastic MC3T3
Cell Behavior**

A Dissertation

Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Kan Wang

August 2011

Copyright © 2011 by Kan Wang

All rights reserved.

Acknowledgements

I would like to express my sincerely thanks to my advisor, Dr. Shanfeng Wang, for his advice, guidance, and support throughout my graduate studies at the University of Tennessee. I want to express my acknowledgement for his being an ideal professional and scholarly role model. I would also like to thank my other committee members, Dr. Benson who has taught me the fundamentals of polymer chemistry and lab method, and who has always had his door open to solve any technical or personal issue encountered during my study at UT, Dr. Kit who has taught me the courses of polymer physics and polymer engineering and has generously provided polarized optical microscopy and thermal stage for my experiments, and Dr. Cui who has provided us the opportunity for biological technique training in her lab with the important help of Dr. Feng Hao. Thanks to Dr. Jingyan Dong and Li Zhang from North Carolina State University for the collaborating work on manufacturing silicon molds, Dr. Xueguang Jiang, Dr. Xiaoming Jiang, and Dr. Bin Zhao in the Department of Chemistry for the help with GPC and contact angle measurements, Dr. Stephen Jesse at Oak Ridge National Laboratory for the help with AFM and film thickness measurements. Also thanks to all my colleagues for their collaboration, discussion, and assistance. Finally, I give my greatest appreciation to my parents Mr. Huayi Wang and Mrs. Lili Zhang and my wife Claudia Liu for their unconditional love and support. I am also deeply grateful to all my relatives and closest friends for their care and support in every possible way when I had illness last year.

Abstract

This dissertation presents material design strategies to investigate cell–biomaterial interactions on specific biocompatible polymers and polymer blends by using mouse pre-osteoblastic MC3T3 cells aiming for potential applications in bone tissue engineering. Chapter 1 reviews some related background knowledge including polymeric biomaterials for tissue engineering, cell–biomaterial interaction, synthetic photo-crosslinkable and degradable polymers, and the effect of surface features on osteoblast cell responses. Chapter 2 presents photo-crosslinkable composites of poly(propylene fumarate) (PPF), an injectable and biodegradable polyester, and methacryl-polyhedral oligomeric silsesquioxane (mPOSS), which has eight methacryl groups tethered with a cage-like hybrid inorganic–organic nanostructure, for bone tissue engineering applications. Blending mPOSS with PPF was found to decrease the viscosity of PPF, expedite photo-crosslinking process, increase tensile modulus and accelerate hydrolytic degradation of crosslinked PPF/mPOSS while it did not significantly alter surface wettability, protein adsorption, and cell response. Chapter 3 demonstrates a polymer blend composed of amorphous PPF and semicrystalline poly(ϵ [epsilon]-caprolactone) (PCL), a widely used biocompatible and biodegradable polymer, in both uncrosslinked and photo-crosslinked forms. Thermal, rheological, mechanical properties, surface hydrophilicity, and morphology can be well controlled by crosslinking density and crystallinity. Distinct cell attachment, spreading, and proliferation have been found to

PPF/PCL blends in the presence or absence of cross-links. Chapter 4 and 5 describe the crystallization induced banded spherulitic morphologies in PPF/PCL blends and PCL homo-blends and their preliminary biological evaluation. Thermal properties, crystallization kinetics, and surface morphology of these blends can be regulated by isothermal crystallization temperature and composition. Surface roughness has been found to play an important role in influencing protein adsorption and cell response. Chapter 6 introduces a newly synthesized biodegradable elastomer, poly(ϵ [epsilon]-caprolactone) triacrylate (PCLTA), with two different molecular weights resulting in distinct mechanical properties at physiological temperature. Using replica molding from silicon wafers, photo-crosslinked PCLTA substrates with concentric micro-grooves have been successfully fabricated. MC3T3 cell attachment, proliferation, and differentiation could be better supported by stiffer substrates while not significantly influenced by micro-groove dimensions. Cell orientation, nuclei shape and localization, mineralization, and gene expression level of osteocalcin have been found to be more significant on narrower micro-grooves when groove depth was 10 μ [mu]m.

Contents

Chapter 1. Introduction	1
1.1 Polymer Biomaterials for Tissue Engineering	2
1.1.1 Tissue Engineering	2
1.1.2 Category of Factors in Determining Cell–biomaterial Interaction	3
1.1.3 Bone Regeneration	4
1.2 Synthetic Photo-crosslinkable and Degradable Polymeric Biomaterials	5
1.2.1 Photo-crosslinking and Network Degradation	5
1.2.2 Poly(propylene fumarate)	6
1.2.3 Other Important Photo-crosslinkable and Degradable Synthetic Polymers	9
1.3 Crystalline Structure and Its Biological Impact	14
1.3.1 Poly(ϵ -caprolactone)	14
1.3.2 Banded Spherulites	15
1.3.3 The effect of Spherulite Roughened Morphology on Cell Responses	16
1.4 Effect of Topographical Features on Osteoblast Cell Behaviors	17
Chapter 2. Methacryl Polyhedral Oligomeric Silsesquioxane (POSS) as a Crosslinker for Expediting Photo-crosslinking of Poly(propylene fumarate): Material Properties and Bone Cell Behavior	20
2.1 Introduction	21
2.2 Experimental Section	23

2.2.1 Materials.....	23
2.2.2 PPF/mPOSS Preparation and Photo-crosslinking.....	24
2.2.3 Characterization of Uncrosslinked and Crosslinked PPF/mPOSS Samples...	25
2.2.4 Contact Angle and Protein Adsorption.....	26
2.2.5 Cell Culture and <i>In Vitro</i> Cytocompatibility.....	27
2.2.6 <i>In Vitro</i> Cell Attachment and Proliferation.....	28
2.2.7 ALP Activity and Calcium Content.....	28
2.2.8 Reverse Transcription (RT) and Real-time Polymerase Chain Reaction (PCR).....	29
2.2.9 Statistical Analysis.....	31
2.3 Results and Discussion.....	32
2.3.1 Structural Characterization of Uncrosslinked PPF/mPOSS.....	32
2.3.2 Thermal Properties of Uncrosslinked PPF/mPOSS.....	32
2.3.3 Viscosity of Uncrosslinked PPF/mPOSS.....	35
2.3.4 Crosslinking Kinetics of PPF/mPOSS.....	37
2.3.5 Thermal Stability of Crosslinked PPF/mPOSS.....	42
2.3.6 Mechanical Properties of Crosslinked PPF/mPOSS.....	45
2.3.7 Degradation of Crosslinked PPF/mPOSS in NaOH Solution.....	47
2.3.8 Surface Hydrophilicity and Protein Adsorption.....	50
2.3.9 Cell Viability, Attachment, Spreading, and Proliferation.....	53

2.3.10 ALP Activity and Calcium Content.....	56
2.3.11 Osteocalcin and ALP Gene Expression.....	57
2.4 Further Discussion.....	60
2.5 Conclusion.....	65
Chapter 3. Distinct Cell Responses to Substrates Consisting of Poly(ϵ-caprolactone) and Poly(propylene fumarate) in the Presence or Absence of Crosslinks.....	67
3.1 Introduction.....	68
3.2 Experimental Section.....	68
3.2.1 Materials.....	70
3.2.2 Preparation of Uncrosslinked and Crosslinked Samples.....	70
3.2.3 Structural Characterization.....	71
3.2.4 Thermal Properties.....	71
3.2.5 Rheological and Mechanical Properties.....	72
3.2.6 Water Contact Angle Measurement and Protein Adsorption.....	73
3.2.7 Cell Culture and <i>In Vitro</i> Cytocompatibility.....	74
3.2.8 <i>In Vitro</i> Cell Attachment, Spreading, and Proliferation.....	75
3.2.9 Statistical Analysis.....	75
3.3 Results and Discussion.....	76
3.3.1 Structural Characterizations.....	76
3.3.2 Rheological Properties.....	76

3.3.3 Gel Fraction.....	81
3.3.4 Thermal Properties.....	81
3.3.5 Mechanical Properties.....	88
3.3.6 Surface Characteristics.....	90
3.3.7 <i>In Vitro</i> Cell Behavior.....	93
3.4 Further Discussion.....	100
3.5 Conclusions.....	105
Chapter 4. Banded Spherulitic Morphology in the Hetero-blends Consisting of Poly(propylene fumarate) and Poly(ϵ-caprolactone) and Interaction with MC3T3 Cells.....	108
4.1 Introduction.....	109
4.2 Materials and Methods.....	112
4.2.1 Materials.....	112
4.2.2 Preparation of Spin-coated PPF/PCL Films.....	113
4.2.3 Characterizations of Isothermally Crystallized PPF/PCL Samples.....	113
4.2.4 Contact Angle Measurement and Protein Adsorption.....	115
4.2.5 Cell Attachment and Proliferation.....	115
4.3 Results and Discussion.....	117
4.3.1 Thermal Properties of Isothermally Crystallized PPF/PCL Blends.....	117
4.3.2 Banded Spherulite Structure.....	121

4.3.3 Crystallization Kinetics.....	123
4.3.4 Surface Morphology of Isothermally Crystallized PPF/PCL ($\phi_{\text{PCL}} = 75\%$) Films.....	127
4.3.5 Protein Adsorption and Cell Study of Isothermally Crystallized PPF/PCL ($\phi_{\text{PCL}} = 75\%$) Films.....	130
4.4 Conclusions.....	134
Chapter 5. Banded Spherulitic Morphology in the Poly(ϵ-caprolactone) Homo-blends and Interactions with MC3T3 Cells.....	136
5.1 Introduction.....	137
5.2 Materials and Methods.....	140
5.2.1 Materials.....	140
5.2.2 Sample Preparation.....	141
5.2.3 Characterizations.....	141
5.2.4 Protein Adsorption.....	143
5.2.5 <i>In Vitro</i> Cytocompatibility, Cell Attachment and Proliferation.....	143
5.2.6 Statistical Analysis.....	145
5.3 Results and Discussion.....	145
5.3.1 Effect of Crystallization Temperature on Thermal Properties.....	145
5.3.2 Spherulitic Morphology.....	151
5.3.3 Crystallization Kinetics.....	156

5.3.4 Surface Morphology of the Samples.....	157
5.3.5 Protein Adsorption and Cell Behavior on the Samples.....	159
5.4 Further Discussion.....	167
5.5 Conclusions.....	170
Chapter 6. Biodegradable Elastomeric Substrates with Concentric Microgrooves for Regulating MC3T3 Cell Behavior.....	172
6.1 Introduction.....	173
6.2 Materials and Methods.....	175
6.2.1 Synthesis and Photo-crosslinking of Poly(ϵ -caprolactone) Triacrylate (PCLTA).....	175
6.2.2 Characterizations of Uncrosslinked and Photo-crosslinked PCLTAs.....	176
6.2.3 Fabrication of Micro-grooved Crosslinked PCLTA Substrates.....	177
6.2.4 Cell culture, <i>In Vitro</i> Cell Attachment, and Proliferation.....	178
6.2.5 Cell Dimensions and Alignment.....	180
6.2.6 Cell Mineralization.....	180
6.2.7 Reverse Transcription (RT) and Real-time Polymerase Chain Reaction (PCR).....	181
6.2.8 Statistical Analysis.....	183
6.3 Results and Discussion.....	183
6.3.1 Characterizations of Uncrosslinked and Photo-crosslinked PCLTA.....	183

6.3.2 Crosslinked PCLTA Micro-grooved Substrates.....	186
6.3.3 Cell Attachment and Proliferation.....	187
6.3.4 Cell Shape and Alignment.....	187
6.3.5 Cell Distribution.....	194
6.3.6 Mineralization.....	197
6.3.7 Osteocalcin Gene Expression.....	201
6.4 Further Discussion.....	201
6.5 Conclusions.....	205
Chapter 7. Conclusion.....	207
References.....	212
Vita.....	230

List of Tables

Table 2.1. Oligonucleotide primer sequences for RT and real-time PCR.....	30
Table 3.1. Thermal properties of uncrosslinked and crosslinked (x) PPF/PCL blends....	82
Table 4.1. Melting temperature (°C) and crystallinity (%) in parentheses for PPF/PCL blends crystallized at different T_c	119
Table 5.1. Melting temperature (°C) and crystallinity (%) in parentheses for PCL80k/2k blends at different T_c	147
Table 5.2. Melting temperature (°C) and crystallinity (%) in parentheses for PCL80k/530 blends at different T_c	148
Table 6.1. Oligonucleotide primer sequences for RT and real-time PCR.....	182

List of Schemes

Scheme 2.1. Photo-crosslinking of PPF/mPOSS.....	22
---	----

List of Figures

Figure 1.1. Chemical structures of PPF and PCL.....	7
Figure 2.1. FTIR spectra of uncrosslinked HPPF/mPOSS blends.....	33
Figure 2.2. DSC curves of uncrosslinked HPPF/mPOSS (a) and LPPF/mPOSS (b) blends.....	34
Figure 2.3. (a) Zero-shear viscosities (η_0) of uncrosslinked PPF/mPOSS blends at temperatures of 25, 37, and 60 °C. (b) Temperature dependence of η_0 for uncrosslinked PPF/mPOSS blends with ϕ_m of 0, 5%, 10%, and 15% plotted as $\ln\eta_0$ versus $1000/T$, where T is absolute temperature in K.....	36
Figure 2.4. (a) Gel fraction of HPPF/mPOSS and LPPF/mPOSS blends after photo-crosslinking for 0.5 to 5 min. (b) Storage modulus G' and viscosity η versus frequency for LPPF/mPOSS blends after photo-crosslinking for 1 min.....	38
Figure 2.5. SEM images of photo-crosslinked (crosslinking time: 3 min) LPPF (a, b) and LPPF/mPOSS with ϕ_m of 10% (c, d) with surface features at the micron scale. The magnification is $\times 30$ for (a) and (c) and $\times 100$ for (b) and (d). The scale bars represent 200 μm in (a) and (c) while 100 μm in (b) and (d).....	40
Figure 2.6. Transparent disks of photo-crosslinked PPF and PPF/mPOSS samples. Upper row: HPPF/mPOSS; lower row: LPPF/mPOSS; ϕ_m of 0, 5%, 10%, and 15% from left to right.....	41
Figure 2.7. TGA thermograms (a, b) and derivative thermogravimetric (DTG) curves (c,	

d) of crosslinked HPPF/mPOSS (a, c) and LPPF/mPOSS (b, d).....	44
Figure 2.8. (a) Representative stress-strain curves from tensile testing and tensile moduli (inset) of crosslinked PPF/mPOSS at 37 °C. (b) Stress and strain (inset) at break of crosslinked PPF/mPOSS at 37 °C.....	46
Figure 2.9. Weight loss of crosslinked HPPF/mPOSS (a) and LPPF/mPOSS (b) samples at different degradation times in 1 N NaOH aqueous solution at 37 °C. The dotted lines in (a) were drawn following the earlier trend to indicate crosslinked HPPF/mPOSS disappeared between 4 and 5 weeks.....	48
Figure 2.10. AFM images of crosslinked PPF/mPOSS disks. Surface roughness is represented by the number in nm with standard deviation in each image.....	51
Figure 2.11. Protein adsorption on crosslinked PPF/mPOSS disks.....	52
Figure 2.12. Mouse MC3T3-E1 cell viability in the presence of crosslinked PPF/mPOSS compared with TCPS as positive (+) control.....	54
Figure 2.13. (a) Fluorescent images ($\times 200$) of MC3T3 cells stained using rhodamine-phalloidin on crosslinked PPF/mPOSS disks at 4 h, days 1, 2, and 4 post-seeding. The scale bar of 100 μm is applicable to all. (b) Normalized cell attachment at 4 h. (c) Cell average area at 4 h. (d) Cell numbers on crosslinked disks at days 1, 2, and 4, compared to cell-seeded TCPS as positive (+) control. *, $p < 0.05$ relative to TCPS and its crosslinked LPPF/mPOSS counterpart with the same ϕ_m at the same culture time.....	55

Figure 2.14. ALP activity and calcium content of MC3T3 cells cultured on crosslinked PPF/POSS disks for (a) 7 and (b) 14 days, compared to cell-seeded TCPS as positive (+) control. *, $p < 0.05$ relative to crosslinked LPPF/mPOSS with the same ϕ_m . #, $p < 0.05$ relative to crosslinked LPPF/mPOSS with other ϕ_m at day 7.....	58
Figure 2.15. Osteocalcin and ALP gene expression normalized by GAPDH in MC3T3 cells cultured on crosslinked PPF/POSS disks for 14 days by (a) real-time and (b) RT PCR analysis. *, $p < 0.05$ relative to crosslinked LPPF/mPOSS with the same ϕ_m	59
Figure 3.1. FTIR spectra of the uncrosslinked (a) and crosslinked (b) samples.....	77
Figure 3.2. Master curves of (a) G' and G'' , (b) viscosity η of PCL and PPF/PCL blends at 140 °C. The temperature dependence of zero-shear viscosity η_0 (c) and horizontal shift factor a_T (d).....	78
Figure 3.3. Gel fractions of crosslinked PPF and PPF/PCL blends (solid squares: theoretical values; open symbols: experimental values).....	83
Figure 3.4. DSC curves of the uncrosslinked (solid curves) and crosslinked (dotted curves) samples recorded in the scans of (a) heating and (b) cooling.....	86
Figure 3.5. TGA thermograms (a, b) and derivative thermogravimetric (DTG) curves (c, d) of the uncrosslinked (a, c) and crosslinked (b, d) samples.....	87
Figure 3.6. Tensile modulus of the uncrosslinked and crosslinked samples at room temperature (solid curves) and at 37 °C (dotted curves).....	89
Figure 3.7. AFM images of crosslinked PPF and PPF/PCL blends as well as PCL.....	91

Figure 3.8. Water contact angles (a) and the concentration of proteins adsorbed (b) on the uncrosslinked and crosslinked samples.....	92
Figure 3.9. MC3T3 cell viability when cultured in the presence of the crosslinked (x) and uncrosslinked samples, indicated by normalizing MTS absorption to the average value of cell-seeded culture polystyrene (TCPS) wells without being exposed to the samples as positive (+) control at 1, 2, and 4 days.....	95
Figure 3.10. Normalized MC3T3 cell attachment 4 h post-seeding on the disks of the uncrosslinked and crosslinked samples with different ϕ_{PPF} , compared with cell-seeded tissue-culture polystyrene (TCPS) as positive control. *, $p < 0.05$ relative to uncrosslinked PPF/PCL ($\phi_{PPF} = 50\%$); +, $p < 0.05$ relative to uncrosslinked PPF/PCL ($\phi_{PPF} = 75\%$); #, $p < 0.05$ relative to uncrosslinked PPF/PCL ($\phi_{PPF} = 75\%$ and 50%).....	96
Figure 3.11. Cell area after 4 h and 1 day of crosslinked PPF and PPF/PCL ($\phi_{PPF} = 75\%$, 50% , and 25%), and uncrosslinked PCL and PPF/PCL ($\phi_{PPF} = 25\%$). *, $p < 0.05$ relative to crosslinked PPF and PCL; #, $p < 0.05$ relative to crosslinked PPF and PPF/PCL ($\phi_{PPF} = 75\%$, 50% , and 25%), and PCL.....	97
Figure 3.12. Fluorescent images (a) and numbers (b) of cells on crosslinked PPF and PPF/PCL ($\phi_{PPF} = 75\%$, 50% , and 25%), PCL and uncrosslinked PPF/PCL ($\phi_{PPF} = 25\%$, 50% , and 75%) at day 1, 2, 4 post-seeding, compared with TCPS as positive control. *, $p < 0.05$ relative to crosslinked PPF/PCL ($\phi_{PPF} = 100\%$ and 75%), PCL, uncrosslinked PPF/PCL ($\phi_{PPF} = 25\%$), TCPS. +, $p < 0.05$ relative to crosslinked PPF/PCL ($\phi_{PPF} = 25\%$),	

PCL, uncrosslinked PPF/PCL ($\phi_{\text{PPF}} = 75\%$ and 50%). #, $p < 0.05$ relative to uncrosslinked PPF/PCL ($\phi_{\text{PPF}} = 50\%$). ^, $p < 0.05$ relative to uncrosslinked PPF/PCL ($\phi_{\text{PPF}} = 75\%$).....	99
Figure 4.1. DSC curves of PPF/PCL blends with ϕ_{PCL} of 25% (a), 50% (b), 75% (c), and 100% (d) crystallized isothermally at different temperatures from 25 to 52 °C.....	120
Figure 4.2. (a) Hoffman-Weeks plots of T_m vs. T_c for PPF/PCL blends crystallized isothermally at different temperatures. (b) Plot of the left-hand side of eq. 2 vs. ϕ_2^2 for PPF/PCL blends.....	122
Figure 4.3. Polarized optical micrographs of the crystalline structures (a) and band width of banded spherulites vs. ϕ_{PCL} (b) for PPF/PCL blends crystallized isothermally at different temperatures.....	125
Figure 4.4. (a) Polarized optical micrographs depicting morphological evolution of the spherulites in the PPF/PCL blends crystallized at 45 °C. The time for photographing was marked in the images. (b) The linear relationship between spherulite diameter and crystallization time for PPF/PCL blends crystallized at 45 °C. (c) Spherulite radial growth rate vs. crystallization temperature for PPF/PCL blends.....	126
Figure 4.5. (a) Polarized optical micrographs of spin-coated PPF/PCL ($\phi_{\text{PCL}} = 75\%$) films prepared from different concentrations and crystallized isothermally at 45 °C. (b) Film thickness of the spin-coated PPF/PCL ($\phi_{\text{PCL}} = 75\%$) as a function of solution concentration.....	128
Figure 4.6. AFM images of the spherulitic morphology (a) and root-mean-square	

roughness of the spherulites (b) on the PPF/PCL ($\phi_{\text{PCL}} = 75\%$) films crystallized isothermally at 25, 37, 45, and 50 °C. (c) SEM images of the banded spherulite formed in PPF/PCL ($\phi_{\text{PCL}} = 75\%$) at 45 °C with magnifications of $\times 300$ (left) and $\times 2000$ (right). (d) AFM height image of banded spherulite on the PPF/PCL ($\phi_{\text{PCL}} = 75\%$) films crystallized at 45 °C and a height profile through A–B.....129

Figure 4.7. Water contact angle and the concentration of proteins adsorbed on the films of PPF/PCL ($\phi_{\text{PCL}} = 75\%$) crystallized at different temperatures.....131

Figure 4.8. Phase-contrast images (a), spreading area (b), and the numbers (c) of MC3T3 cells on the PPF/PCL ($\phi_{\text{PCL}} = 75\%$) films crystallized at 25, 37, and 45 °C after culturing for 4 h, 1, 2, and 4 days. The scale bar of 100 μm is applicable for all the images in (a). Cell area in (b) was examined at 4 h and day 1 only. The positive control in (c) is tissue culture polystyrene.....133

Figure 5.1. DSC curves of PCL80k (a) and PCL80k/2k blends with $\phi_{80\text{k}}$ of 75% (b), 50% (c), and 25% (d) isothermally crystallized at various temperatures from 25 to 52 °C...149

Figure 5.2. DSC curves of PCL80k/530 blends with $\phi_{80\text{k}}$ of 75% (a), 50% (b), and 25% (c) isothermally crystallized at various temperatures from 25 to 50 °C.....150

Figure 5.3. Hoffman-Weeks plots of T_m vs. T_c for PCL80k and its blends with PCL2k (a) and PCL530 (b) isothermally crystallized at various temperatures.....152

Figure 5.4. Polarized optical micrographs of the crystalline structures (a) and the band width of banded spherulites (b) formed in PCL80k/2k blends when they were crystallized

at various temperatures.....	154
Figure 5.5. Polarized optical micrographs of the crystalline structures (a) and the band width of banded spherulites (b) formed in PCL80k/530 blends when they were crystallized at various temperatures.....	155
Figure 5.6. Crystallization kinetics. (a) Polarized optical micrographs depicting the morphological evolution of the spherulites in PCL80k/2k ($\phi_{80k} = 50\%$) at different crystallization temperatures (T_c). The time for photographing was indicated. (b) Spherulite radial growth rate at different T_c for PCL80k and its blends with PCL2k and PCL530.....	158
Figure 5.7. AFM images of a hot-compressed disk (a) and spin-coated thin films of PCL80k crystallized at 25 °C (b) and 52 °C (c,d), and a hot-compressed disk (e) and spin-coated thin films of PCL80k/2k ($\phi_{80k} = 75\%$) crystallized at 25 °C (f) and 50 °C (g).....	160
Figure 5.8. AFM height images of banded spherulites obtained on the spin-coated thin films of PCL80k crystallized at 52 °C (a), PCL80k/2k ($\phi_{80k} = 75\%$) crystallized at 50 °C (c). A–B height traces (b, d) and root-mean-square (rms) roughness (R_{rms}) (e).....	161
Figure 5.9. Concentration of serum proteins adsorbed on the flat, hot-compressed disks and spin-coated films of PCL80k and PCL80k/2k ($\phi_{80k} = 75\%$) blend isothermally crystallized at different temperatures.....	164
Figure 5.10. Normalized MC3T3 cell attachment at 4 h (a) and number of MC3T3 cells	

at days 1, 2, and 4 post-seeding (b) on the flat hot-compressed disks and spherulite-roughened films of PCL80k and its blend with PCL2k ($\phi_{80k} = 75\%$), compared with cell-seeded TCPS as the positive (+) control. * $p < 0.05$ relative to the PCL80/2k ($\phi_{80k} = 75\%$) counterparts and TCPS. ^ $p < 0.05$ relative to PCL80k thin films crystallized at 37 and 52 °C. # $p < 0.05$ relative to PCL80/2k ($\phi_{80k} = 75\%$) crystallized at 37 and 50 °C.....165

Figure 5.11. (a) MC3T3 cells on PCL80k thin films crystallized at 52 °C after culturing for 4 days demonstrated by merging a DAPI-stained fluorescent image and a POM image together. (b) Percentage of cell nuclei located in or close to the valley of the banded spherulites formed in PCL80k and PCL80k/2k ($\phi_{80k} = 75\%$) when they were crystallized at 52 and 50 °C, respectively. Two different cell densities were used.....166

Figure 6.1. (A) Chemical structure of PCLTA. (B) Schematic difference between PCLTA7k and 10k before and after crosslinking. (C) DSC curves for uncrosslinked and crosslinked PCLTAs. (D) Representative stress-strain curves of crosslinked PCLTAs at 37 °C.....185

Figure 6.2. (A) Schematic micro-fabrication of silicon wafers with a pre-designed pattern and preparation of micro-grooved substrates of photo-crosslinked PCLTA via replica molding: Photolithography creates surface features on silicon substrate (a), dry etching (b), obtain silicon mold (c), pour polymer onto the mold and UV curing (d), 2D scaffold/ mold separation (e), fabricate scaffold with designed pattern (f). (B) SEM images of

typical micro-fabricated silicon wafers with magnifications of $\times 20$ (left) and $\times 1200$ (right).....	188
Figure 6.3. SEM images of micro-grooved PCLTA substrates with four different groove widths. Upper row: PCLTA7k; lower row: PCLTA10k. The magnification is $\times 100$ for all the images. The scale bar represents 200 μm	189
Figure 6.4. SEM images of micro-grooved PCLTA substrates (groove width: 16.1 μm) with three different groove depth. Upper row: PCLTA7k; lower row: PCLTA10k. The magnification is $\times 500$ for all the images. The scale bar represents 40 μm	190
Figure 6.5. (A) Fluorescent images of MC3T3 cells stained using rhodamine-phalloidin and DAPI on flat and micro-grooved PCLTA substrates (depth: 10 μm) with four different width at 4 h post-seeding. Upper row: PCLTA7k; lower row: PCLTA10k. The scale bar represents 100 μm . (B) Number of MC3T3 cells at 4 h, days 1, 2, and 4 post-seeding. Cell length (C), spreading area (D), and alignment (E) at 4 h post-seeding.....	193
Figure 6.6. (A) Fluorescent images of cell nuclei on the micro-grooves (depth: 10 μm) with different width (a-d), flat surface (e), and intersection between flat and micro-grooved surfaces (f) of PCLTA10k at day 4 post-seeding. The scale bar represents 100 μm . (B) Shape index of nuclei on flat and micro-grooved PCLTA10k substrates at day 4 post-seeding calculated from the images in (A). (C) The percentage of cell nucleus located in the micro-grooves of PCLTAs as a function of culture time from 4 h to day 4.....	195

Figure 6.7. (A) Rhodamine-phalloidin stained fluorescent images on PCLTA10k micro-grooved substrates (width: 7.5 μm) with the groove depth of 10 (a), 1 (b), and 0.2 μm (c) at 4 h post-seeding and DAPI stained nuclei images on PCLTA10k micro-grooves (width: 16.1 μm) with the depth of 10 (d), 1 (e), and 0.2 μm (f) at day 4 post-seeding. The scale bar represents 100 μm . (B) Cell alignment on micro-grooved PCLTA substrates (depth: 1 μm) at 4 h post-seeding. (C) The percentage of cell nucleus located in PCLTA10k micro-grooves with the groove depth of 10 and 1 μm at day 4 post-seeding.....196

Figure 6.8. (A) Alizarin red S stained images of PCLTA10k (width: 16.1 μm) at 1 week (a), 2 weeks (b), and PCLTA7k (width: 16.1 μm) at 2 weeks (d); Harris hematoxylin solution and eosin Y solution stained image of PCLTA10k (width: 16.1 μm) at 2 weeks (c); images taken on the intersection between flat and micro-grooved surfaces with the groove width of 7.5 (e), 16.1 (f), 44.2 (g), and 91.2 μm (h) at 2 weeks. (B) Quantification of Alizarin red S extracted from flat and micro-grooved PCLTAs with the depth of 10 μm at 2 weeks. Negative control is the pure cetylpyridinium chloride solution. * $p < 0.05$, relative to the counterpart of PCLTA7k, # $p < 0.05$ relative to flat and micro-grooved PCLTA10k with the width of 44.2 and 91.2 μm , ^ $p < 0.05$ relative to flat PCLTA10k, + $p < 0.05$ relative to micro-grooved PCLTA7k with the width of 91.2 μm 199

Figure 6.9. Osteocalcin gene expression normalized by GAPDH in MC3T3 cells cultured on flat and micro-grooves of PCLTA10k and flat PCLTA7k substrate for 14 days by real

time (A) and RT PCR (B) analysis.....	200
---------------------------------------	-----

Chapter 1. Introduction

1.1 Polymer Biomaterials for Tissue Engineering

1.1.1 Tissue Engineering

Tissue engineering, an interdisciplinary field, integrates the knowledge from physics, chemistry, engineering, material sciences, biology and clinical medicine to develop biological substitutes for restoring, maintaining, or improving the functions of tissue [1,2]. Due to its biocompatibility and controllable mechanical properties, macro- or micro-structure and the degradation rate, polymeric biomaterial becomes a promising candidate for fabricating three-dimensional (3D) scaffold for tissue engineering which can support cell colonization, migration, proliferation, and differentiation, and often guides the development of the required tissue [3-5]. As these polymeric biomaterials gradually degraded *in vivo*, the growing tissue could replace them eventually. The development of 3D culture system is complex and often requires precise tailoring of the material environment for a given cell type. Essentially, tissue engineering requires non-cytotoxic and biodegradable polymeric materials with biocompatibility, feasibility of fabrication, and controllability of mechanical properties to satisfy diverse clinical needs such as soft and hard tissue replacement. During the past few decades, numerous polymeric biomaterials, ranging from loosely crosslinked and hydrophilic soft hydrogels to highly crosslinked and hydrophobic rigid networks, have been developed for tissue engineering applications. These naturally derived or chemically synthesized monomers and macromers can be further modified with various functionalities via different synthetic

chemistry and subsequently polymerized into polymeric degradable networks to satisfy the requirements for a variety of biomedical applications.

1.1.2 Category of Factors in Determining Cell–biomaterial Interaction

Cell/tissue–biomaterial interactions are critical in understanding the material design criteria for tissue engineering as well as clinical considerations [6-8]. In the past several decades, pioneer scientists have achieved great understanding on the roles of the surface physicochemical characteristics of biomaterials in determining cell behaviors [6-8]. These factors can be divided into three major categories: surface chemistry, surface morphology, and surface mechanics [6-8]. The category of surface chemical factors includes different functional groups, surface energy or hydrophilicity, protein adsorption, and surface treatments with charges and/or extracellular matrix (ECM) proteins [6-10]. Topological features include surface patterns and surface roughness at both micro and nanometer scales, which can be used to control cell spreading, orientation, and proliferation through the contact guidance effect and different surface area because they are comparable to the dimensions of cell body, integrin clusters, and individual proteins [6-8]. Mechanical factors such as surface stiffness and hydrodynamic shear stress have been revealed to be critical in influencing cell responses as the actin–myosin interactions served as probing forces can activate stress-sensitive ion channels or protein conformational changes to affect the stability of focal adhesion sites [6-8,11-13]. These surface physicochemical characteristics are correlated together to influence cell behaviors.

1.1.3 Bone Regeneration

Bone regeneration is needed in diverse clinical scenarios such as resection of primary and metastatic tumors, bone loss after skeletal trauma, total joint arthroplasty with bone deficiency, spinal arthrodesis, and trabecular voids [14,15]. Although autogenic bone grafts are commonly used, they have limitations such as additional surgery, donor site morbidity, and limited availability [14,15]. As promising substitutes for autologous or allograft bone, polymeric biomaterials have been developed with desirable properties in biocompatibility, controlled degradability, mechanical integrity, osteoconductivity, sterilizability, and handling characteristics [14,15]. For example, poly(methyl methacrylate) (PMMA) bone cements are traditionally used in orthopedic surgeons to fix various implant prostheses [16]. Generally, PMMA chains are mixed with methyl methacrylate monomer to form viscous polymer solutions which can be injected and polymerized into networks via redox, thermal, or photo initiation *in situ*. Although these materials have excellent mechanical properties, they are nondegradable which could result in complications with tissue healing such as limiting blood flow. Because bone has the capability of healing itself, the treatment of skeletal defects can benefit from degradable polymeric biomaterials formed *in situ* which can temporarily restore the function and then subsequently degrade with a tailored rate comparable to the healing process.

1.2 Synthetic Photo-crosslinkable and Degradable Polymeric Biomaterials

1.2.1 Photo-crosslinking and Network Degradation

Photo-crosslinking is the radical polymerization of multifunctional monomers which requires the use of photoinitiators that can dissociate into radicals upon the absorption of light energy [17]. The prerequisite for photo-initiated crosslinking is the presence of unsaturated chemical bonds like acrylate and methacrylate groups. Commonly used photon-energy sources are long wave ultraviolet or blue visible radiation and the initiation rate mostly depends on parameters such as light intensity, initiator concentration, and initiator efficiency. Photo-crosslinking has many advantages over other covalently crosslinking techniques, such as thermal or redox initiated crosslinking, for biomedical applications [18-20]. The primary one is the spatial and temporal control of the process which could result in tunable polymerization exotherms and time of gelation. Since the initial materials are normally liquid, the injection system can be easily loaded in complex shapes and subsequently cured into polymer products with exactly designed dimensions without additional modification of implants. It also provides rapid polymerization rate at low temperatures thus reducing possible denaturation of biological compounds.

Biomedical polymers can be classified as nondegradable and biodegradable depending on their behavior after an implant or when in contact with biological fluids [17]. Degradation can occur through either passive hydrolytic cleavage or biologically active process in an aqueous or an enzymatic environment, respectively. For the

photo-crosslinkable biodegradable polymers, the networks formed via light in presence of photoinitiator typically can hydrolytically or enzymatically cleave into products of segments of the starting polymer, original core molecule, the degradable units, or the kinetic chains formed during the free-radical polymerization of the photoreactive groups depending on the nature and location of degradable units and reactive groups. The degradation rate can be strongly influenced by macromer structure, backbone hydrophobicity, and network crosslinking density [17]. For example, the higher crosslinking density resulting from the higher concentration or lower molecular weight of the macromer generally can lead to longer network degradation time because of slower water diffusion and less bonds that are necessarily cleaved to break the network.

1.2.2 Poly(propylene fumarate)

Poly(propylene fumarate) (PPF) (Figure 1.1) is an amorphous, unsaturated linear polyester and an excellent injectable biomaterial that can be crosslinked into scaffolds for load-bearing purposes in bone tissue engineering applications [21]. The molecular weight of PPF can be controlled by varying the reaction time and/or temperature in its two-step polycondensation of diethyl fumarate (DEF) and propylene glycol [22-24]. The physical properties of PPF such as glass transition temperature (T_g) and zero-shear viscosity (η_0) demonstrate strong molecular weight dependence [24]. PPF can be thermally crosslinked or photo-crosslinked through its fumarate double bonds and can be degraded into non-toxic degradation products by simple hydrolysis of the ester bonds [25,26]. The

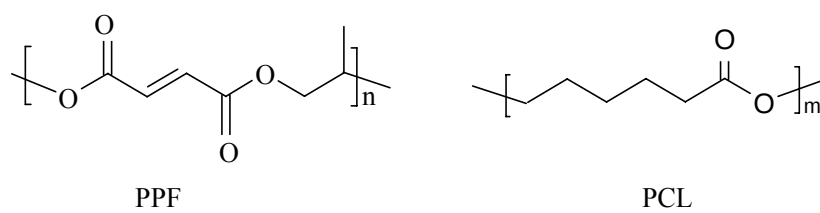


Figure 1.1. Chemical structures of PPF and PCL.

degradation time depends on polymer characteristics such as molecular weight, type of crosslinker, and crosslinking density [23].

Various fabrication methods have been employed to manufacture three-dimensional (3D) porous crosslinked PPF scaffolds with controlled pore size, porosity, and geometry for bone repair and regeneration [26-33]. In stereolithography, PPF needs to be dissolved in DEF to prepare photo-crosslinkable PPF/DEF resin, whereas DEF with a high boiling point of ~ 192 °C can participate in the crosslinking of PPF [28-33]. Both PPF molecular weight and concentration can be used to regulate the resin viscosity, which has to be sufficiently low (< 1.8 Pa.s) for ease of operation [28]. Recently a deposition and cure method is being developed in our collaborative research groups to manufacture porous scaffolds using crosslinkable polymer resins. In these methods, the resin reactivity should be adequately high for instant curing upon exposure to UV light, although this goal can also be reached by increasing the UV dosage.

To regulate material properties and cell behavior, numerous monomers or polymers have been copolymerized or blended with PPF and crosslinked into controllable networks [34-39]. Inorganic fillers such as hydroxyapatite (HA) nanoparticles, β -tricalcium phosphate, calcium sulfate, and single-walled carbon nanotubes have been incorporated with PPF into crosslinked composites [40-46]. As the resin for scaffolding, PPF/DEF have composition dependent mechanical properties after crosslinking and an intermediate composition of $\sim 75\%$ was found to be optimal in achieving maximal tensile modulus and

fracture strength [35]. PPF was also blended and crosslinked with another crosslinkable polymer poly(ϵ -caprolactone) fumarate (PCLF) or poly(ϵ -caprolactone) diacrylate (PCLDA) to obtain a wide range of mechanical properties through modulating the crosslinking density, which was consequently used to regulate cell behavior and satisfy diverse needs in hard and soft tissue replacements [36,39]. In these polymer networks with crystalline domains confined by the crosslinks, the crystallinity and melting temperature of PCL are significantly reduced by increasing the crosslinking density through the composition of PPF. In our recent study, polyethylene glycol monoacrylate (mPEG) with one reactive end group was incorporated with PPF networks as hydrophilic dangling chains to improve surface wettability [38]. Photo-crosslinked PPF/mPEG networks demonstrated parabolic dependence of mouse MC3T3 cell attachment, proliferation, and differentiation on the network composition because improved surface hydrophilicity at low densities of tethered PEG chains could enhance cell attachment and proliferation while more tethered PEG chains were repulsive to proteins and cells [38].

1.2.3 Other Important Photo-crosslinkable and Degradable Synthetic Polymers

Water-soluble poly(ethylene glycol) (PEG) have been widely studied and used for numerous biomedical applications due to its well-known low toxicity and good biocompatibility. It has some unique characteristics such as extremely hydrophilic and protein resistant. PEG with mono-, or multi- reactive hydroxyl groups can be simply modified to different PEG macromers with unsaturated moieties via various synthetic

reactions in order to form distinct photo-crosslinked networks. For example, vinyl segments can be incorporated in to PEG backbone using acryloyl chloride or methacryloyl chloride in the presence of triethylamine (TEA) to form PEG acrylate or methacrylate [47]. Poly(ethylene glycol fumarate) (PEGF), a PEG derivative containing the cross-linkable moiety, has been synthesized by the reaction between PEG and fumaryl chloride in presence of triethylamine (TEA) and the physical properties can be easily tailored by changing PEG molecular weight [48]. The amphiphilic copolymer PEGF-*co*-PCLF can be prepared by connecting the units of hydrophobic PCL or hydrophilic PEG precursor together with fumarate segments to control over hydrophilicity, swelling, and mechanical properties [49]. Nitric oxide (NO) donors containing PEG hydrogel has been developed and the released NO from hydrogel in physiological condition can successfully inhibit smooth muscle cell proliferation and platelet adhesion which lead to the potential applications for reducing the thrombosis and restenosis [50]. Highly branched and regularly structured biodendritic PEG-based macromolecules have been successfully prepared with low solution viscosities, high solubility, and multiple end groups for functionalization and subsequently can form hydrogels via photoinitiation resulting in high crosslinking density, proper mechanical property, and excellent biocompatibility [51-53].

Crosslinkable poly(ϵ -caprolactone fumarate) (PCLF) has been synthesized by incorporating unsaturated fumarate segments into PCL blocks [36]. Potassium carbonate

(K₂CO₃), a substitute for traditionally-used triethylamine (TEA), has been used as the proton scavenger in the esterification of PCL diol precursors with hydroxyl end group to avoid colored, watersoluble cytotoxic complexes formed from side reaction between TEA and unsaturated acyl chlorides [54]. Crosslinked PCLF can degrade through the hydrolysis of its ester linkages in both PCL blocks and fumarate segments under physiological conditions and its mechanical properties can be well tuned by crystallinity and crosslink density through monomers with different molecular weights [55]. PCLF have been employed to prepare nanocomposites with hydroxyapatite nanoparticles with controllable material properties for bone-tissue engineering applications, and fabricate single- or multi-channel nerve conduits with excellent biocompatibility and tailorable physical properties for guiding axon growth in peripheral nerve regeneration [56,57]. Besides, PPF has been blended with PCLF to form hybrid polymeric networks via photo-crosslinking resulting in a progressive change from flexible and weak materials to strong and stiff ones which can lead to potential applications for both soft and hard tissue replacements [36].

Hydrolytically degradable poly(β -amino ester) (PBAE) can be readily synthesized via the conjugate addition of primary or secondary amines to diacrylates with no byproducts resulting in linear macromers containing both esters and amines in their backbones and subsequently UV crosslinked to form networks for the development of biomedical applications such as gene delivery and 3D scaffolds [58-60]. A combinatorial

library of diverse photocrosslinkable and degradable PBAE macromers has been achieved using different functionalized amines or diacrylates [61]. The photocured PBAEs can be degraded in NaCl PBS at 37 °C with the wide degradation time ranging from less than one day to more than four months and have the elastic modulus E tested by a rapid nanoindentation technique ranging from ~ 4 to ~ 350 MPa, which can be also influenced by the molecular weight and branching degree of PBAE macromers [61,62].

Poly(vinyl alcohol) (PVA), a water-soluble and biodegradable synthetic polymer, is known as an excellent biomaterial for diverse applications, such as contact lenses, tendon repair, and scaffolds for tissue engineering. The multiply pendant hydroxyl groups on the PVA backbone can be modified with various functional groups containing unsaturated segments and subsequently form hydrogel network via UV initiation that has many advantageous such as high water content and tissue-like elasticity. For instance, acrylate, methacrylate, and acrylamide groups have been successfully incorporated into PVA using acrylic and methacrylic acid and 2-vinyl-4,4-dimethyl-azlactone to obtain water-soluble polymers [63]. By photo-crosslinking, the transparent networks can be formed within seconds with good mechanical properties and high water content (60–80%) leading to the potential application as contact lenses material [63]. Hybrid polymeric network and copolymer that contain photo-crosslinkable modified PVA have been also prepared and then photopolymerized to produce the hydrogels with controllable degradation, mechanical, and biological properties for biomedical applications [64-68].

A class of polyanhydrides (PA) has been developed which can be cured photochemically to produce form high-strength and surface degrading networks for orthopedic and drug delivery applications. Dimethacrylated sebacic (MSA), 1,6-bis (carboxyphenoxy) hexane (MCPH), and 1,3-bis (carboxyphenoxy) propane (MCPH) monomers have been synthesized by converting dicarboxylic acid to an anhydride by heating in excess of methacrylic anhydride while trimethacrylated tricarballic acid (MTCA) and methacrylated pyromellitylimidoalanine (MPMA-ala) can be prepared similarly with more monomer functionality and rigid segment in backbone resulting in higher crosslinking efficiency, density, and mechanical strength [69-71]. Monovinyl monomers of methacrylated stearic acid (MStA) and methacrylated cholesterol (MC), obtained by reaction of stearic acid and cholesterol with methacryloyl chloride in presence of triethylamine, have been grafted onto polyanhydride networks in order to tailor surface degradation rate and morphology [72]. The highly crosslinked polyanhydrides' networks degrade via surface erosion mechanism that only allows minimal penetration of water into the polymer resulting in higher degree of mechanical property maintained during the hydrolytic degradation [17]. The degradation time, depending on the overall hydrophobicity in the polymer network, can be varied from 3 days (MSA) to more than one year (MCPH) and a series of copolymers or hybrid polymeric networks containing these photocrosslinkable polyanhydrides have been prepared to tune the degradation rate [73,74].

1.3 Crystalline Structure and Its Biological Impact

1.3.1 Poly(ϵ -caprolactone)

Poly(ϵ -caprolactone) (PCL) (Figure 1.1) is a well-known biodegradable polymer with excellent biocompatibility and processability which has been approved by U.S. Food & Drug Administration (FDA) for biomedical applications [75,76]. PCL can degrade through hydrolysis of its ester linkages in physiological conditions [75,76]. It is a semicrystalline polyester with a glass transition temperature of $-60\text{ }^{\circ}\text{C}$ and molecular-weight-dependent melting point as well as crystallinity. The melting temperature of PCL increases asymptotically with the molecular weight to a constant value of $\sim 55\text{ }^{\circ}\text{C}$ [77]. However, the crystallinity of PCL has a maximum when the molecular weight is $\sim 20,000\text{ g/mol}$ [77]. In addition, the entanglement molecular weight (M_e) for PCL has been obtained to be $\sim 3000\text{ g/mol}$ from both molecular weight dependence of viscosity and plateau modulus [78]. In order to achieve tailorable material properties for diverse tissue engineering applications and regulating cell behaviors, PCL has been blended with other polymeric biomaterials such as polysaccharides, poly(D,L-lactic-*co*-glycolic acid) (PLGA), and chitosan [79-82]. Photo-crosslinkable and biodegradable poly(ϵ -caprolactone fumarate)s (PCLFs) and PCL acrylates (PCLAs) have been synthesized from oligomeric PCL diols or triols with different molecular weights [49,83].

1.3.2 Banded Spherulites

Spherulites have been noted as the characteristic morphological appearance in most crystalline polymer which are formed during polymer crystallization due to the packing of polymer chains and examined by using a polarized optical microscope (POM) [84]. Banded spherulites are spherulites showing regular concentric bands along the radiating direction because of polymer lamellar twisting from the unbalanced surface stress, which have been found in some crystalline polymers, such as polyethylene (PE), polyethylene terephthalate (PET), poly(3-hydroxybutyrate) (PHB), and PCL [85-87]. It has been found that the banded spherulites of PCL can be obtained at a high crystallization temperature such as 50 °C or above which is very close to its T_m [88,89]. The presence of banded spherulites has also been observed in some miscible blends of PCL with polymethacrylates, poly(styrene-*co*-acrylonitrile) (SAN), poly(vinyl chloride) (PVC), poly(vinyl methyl ether) (PVME), and in some diblock or triblock copolymers consisting of PCL segments such as polybutadiene-*co*-polycaprolactone (BC), polystyrene-*co*-polybutadiene-*co*-polycaprolactone (SBC), and poly(propylene fumarate-*co*-caprolactone) [P(PF-*co*-CL)] at proper crystallization temperatures as well as certain PCL compositions [37,88-97]. For example, oligomeric and amorphous PPF was copolymerized with hydroxyl-terminated semicrystalline PCL to form a series of novel cross-linkable and biodegradable multiblock P(PF-*co*-CL) copolymers via a three-step polycondensation resulting in strong PCL composition dependent physical

properties [37]. Due to the PCL lamellar twisting along the radial direction during crystal growth induced by the strong surface stress and impeded crystal growth rate, the banded patterns were formed in the PCL spherulites at room temperature when ϕ_{PCL} was 87% in the copolymer [37].

1.3.3 The effect of Spherulite Roughened Morphology on Cell Responses

Polymers are widely used in engineering biomedical devices for tissue engineering applications and the method used for processing these polymers plays an important role in determining the material properties and subsequently influencing the cellular response [98]. For crystalline polymers, the surface can be roughened by the spherulites formed during polymer crystallization. For example, investigations of the effects of processing methods have focused on the surface characteristics and biological responses of poly(L-lactic acid) (PLLA), a crystalline and biodegradable polymer widely used in tissue engineering applications [99-101]. By using quench and isothermal crystallization processing methods, amorphous and crystalline PLLA substrates were prepared to examine the effect of crystallinity on biological responses [99]. It has been found that hepatocyte adhesion, proliferation and differentiation were enhanced on crystalline PLLA substrates and 3T3 fibroblast cells showed higher proliferation rate on amorphous PLLA samples than crystalline ones [99]. Gradients of polymer crystallinity were also achieved on PLLA films by using different annealing temperatures increasing from 45 to 100 °C resulting in increased surface root-mean-square (rms) roughness ranging from 0.5 to 13

nm [100]. It has been shown that the proliferation rate of MC3T3-E1 osteoblastic cells on the smooth films of PLLA is much greater than that on the rough surfaces and a monotonic variation in rate was observed as a function of roughness [100]. Amorphous poly(D,L-lactic acid) (PDLLA) was blended with crystalline PLLA to achieve a wider gradient of roughness resulting in smooth surface on PDLLA-rich regions and rough surface on PLLA-rich regions and the maximum rms roughness of ~ 70 nm value obtained at ϕ_{PLLA} of $\sim 70\%$ [101]. Similarly, it has been observed that MC3T3 cell adhesion was similar on all the PDLLA-PLLA blends while cell proliferation was faster on the PDLLA-dominant sample ($\phi_{\text{PLLA}} = \sim 30\%$) with smoother surface (rms roughness: ~ 11 nm) [101]. However, the mechanism of this cell response to crystallization-induced surface roughness is still unclear and the results presented in the literature for cell adhesion as well as proliferation on non-structured randomly rough surfaces remain controversial.

1.4 Effect of Topographical Features on Osteoblast Cell Behaviors

Surface morphological factor, including surface roughness and surface patterns at the micron and nanometer scales, is one of the major categories to influence cell-substrate interactions [6-8]. Established techniques, such as lithographic-based methods, polymer demixing, electrochemical etching, and molecular self-assembly, have enabled the fabrication of substrates with various controllable topographical features exhibiting short and long range orders [102]. With the help of these advance microfabrication techniques,

many studies have been performed to create substrates with nano- and micro-topographical patterns, such as pits, pillars, pores, columns, grooves, random rough surface, in order to mimic *in vivo* microenvironments and regulate cell behavior [103-108]. The cells can sense, communicate, and respond to the signal from these topographical features and subsequently they could evidently affect *in vitro* cell adhesion, cytoskeleton organization, migration, proliferation, differentiation, and gene expression [103-108]. When cells are seeded on microgrooved substrates, they could align, grow, or travel parallel to the groove direction, which is known as “contact guidance” and this response has been demonstrated to be cell-type dependent [109-112].

Researchers have studied “contact guidance” effect on osteoblast-like cells using microfabricated metallic and polymeric substrates to understand the interactions between bone tissue and implants and to further improve cellular functions and tissue growth [113-118]. Cells could be aligned or migrate toward the microgroove direction when both depth and groove width mostly ranged either on the microscopic or nanoscopic level and the percentage of oriented cells is strongly dependent on the feature size. In addition, it has been reported that the differentiation and mineralization of osteoblasts can be influenced by surface features on various materials [114,119-122]. For example, rough-textured and porous-coated titanium surfaces can enhance both the synthesis and mineralization of the extracellular matrix [119]. Assessed by radiography and computer image processing at the implant interface, *in vivo* report also demonstrated that

mineralization occurred often on grooved, pitted, and polished rarely on smooth titanium surfaces that were implanted in rats using [120,121]. Calcium deposits from rat bone marrow cells (rBMCs) can be clearly seen inside the denser groove spacing compared with smooth polystyrene substrates from transmission electron microscopy observation [114]. The rBMCs showed higher alkaline phosphatase (ALP) activity on grooved surfaces of polylactic acid with a depth of 1 μm and a width of 1 and 2 μm than non-grooved substrates [122]. Because bone grafts or implants have been used to treat destructive bone diseases such as osteoporosis, osteoarthritis, and bone tumor, it is crucial to optimize the surface geometrical feature dimensions for promoting osteoblast cell positioning, alignment, and differentiation.

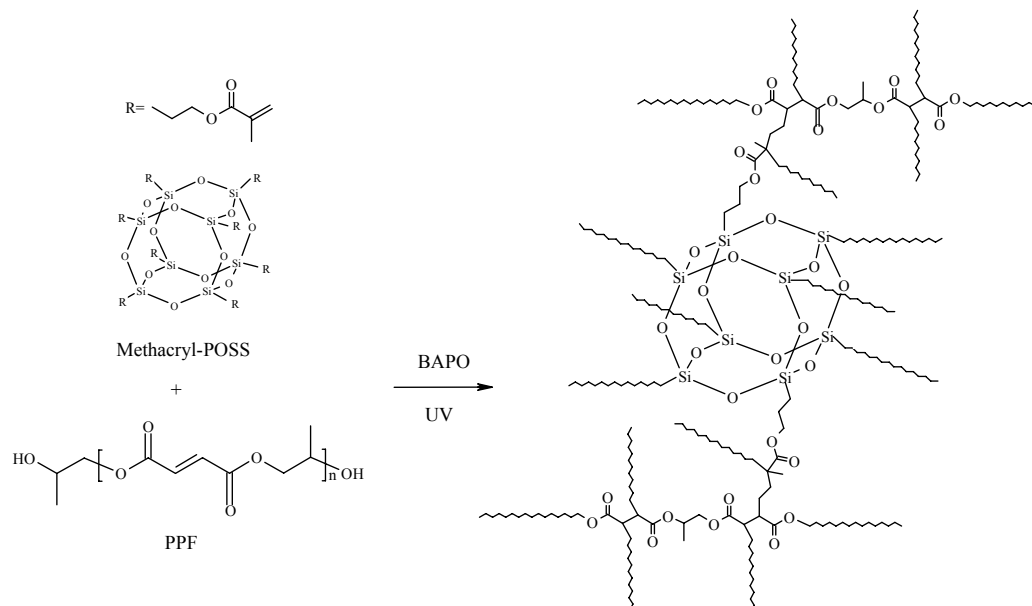
**Chapter 2. Methacryl Polyhedral Oligomeric Silsesquioxane (POSS) as
a Crosslinker for Expediting Photo-crosslinking of Poly(propylene
fumarate): Material Properties and Bone Cell Behavior**

2.1 Introduction

Polyhedral oligomeric silsesquioxane (POSS) has a cage-like hybrid inorganic–organic structure composed of a silicon–oxygen framework with a diameter of approximately 1.5 nm when the vertex (*R*) groups are included [123-127]. The *R* groups can be easily functionalized with non-reactive or reactive organic substitution [123-131]. It has been reported that incorporating powder-like POSS cages with polymers can achieve improved thermal and mechanical properties [128-131]. As illustrated in Scheme 2.1, methacryl-POSS (mPOSS) or methyl methacrylate-POSS used in this study is a polydisperse and oily liquid at room temperature. mPOSS can polymerize into a transparent, light colored, and self-stand hybrid material. Nanocomposites have been prepared by crosslinking mPOSS or other polymerizable POSS with polymers such as polyimide, polymethyl methacrylate (PMMA), trifunctional furan compound, and methylsilicone resin [132-143]. Because they are non-toxic and cytocompatible, POSS-based polymeric nanocomposites can be suitable for biomedical applications [126,127]. In particular, POSS with one or eight reactive vertex groups has been incorporated with methacrylate-based hybrid dental materials to suppress the volume shrinkage and enhance the biocompatibility [141-143]. POSS-containing nanocomposites have been also developed and their biocompatibility has been evaluated for potential cardiovascular applications [144-147]. Although POSS has been studied extensively, little knowledge is known for using it to develop novel injectable biomaterials for bone tissue engineering applications.

We presently blend PPF with mPOSS and crosslink them via photo-initiation to achieve novel injectable, crosslinkable, non-cytotoxic, and biodegradable polymeric

Scheme 2.1. Photo-crosslinking of PPF/mPOSS



systems with improved processability and tunable bulk and surface characteristics for objectives in bone repair and regeneration. Two PPF samples, one with a higher molecular weight (HPPF) and one with a lower molecular weight (LPPF), were blended with mPOSS at various mPOSS compositions. Because mPOSS molecules are nanometer-sized cages with eight crosslinkable end groups, we anticipate that it can decrease the viscosity of PPF and accelerate crosslinking of PPF, which are two critical issues in material processing and scaffold fabrication. Both uncrosslinked and crosslinked PPF/mPOSS samples have been characterized extensively on their structural, thermal, rheological, mechanical properties, surface wettability, and capability of adsorbing serum proteins from cell culture media. Two important variables, the molecular weight of PPF and the composition of mPOSS (ϕ_m), have been studied in determining the crosslinking process and material properties of PPF/mPOSS. Further, mouse pre-osteoblastic MC3T3 cell studies have been performed to evaluate *in vitro* biocompatibility of these hybrid polymeric networks in bone tissue engineering applications and to find the controllability of bone cell behavior by varying surface characteristics.

2.2 Experimental Section

2.2.1 Materials

Gel permeation chromatography (GPC) was conducted at room temperature on an integrated GPC system (PL-GPC20, Polymer Laboratories, Inc.) with a refractive index detector to determine the weight-average molecular weight (M_w) and number-average molecular weight (M_n), and polydispersity (M_w/M_n) of PPF. The data were processed

using Cirrus GPC/SEC software (Polymer Laboratories). Tetrahydrofuran (THF) was used as the eluent with a flow rate of 1.0 mL/min and standard monodisperse polystyrene samples (Polymer Laboratories) were used for calibration. Two PPF samples with M_n , M_w of 3100, 5200 g.mol⁻¹ for HPPF and M_n , M_w of 850, 1150 g.mol⁻¹ for LPPF were synthesized in our laboratory as described earlier [24,37,38,148]. mPOSS was purchased from Hybrid Plastics (Hattiesburg, MS) and used as received.

2.2.2 PPF/mPOSS Preparation and Photo-crosslinking

In discussion, pure PPF and mPOSS are included as PPF/mPOSS ($\phi_m = 0$) and PPF/mPOSS ($\phi_m = 100\%$), respectively. As described in Scheme 2.1, 75 μ L solution of photo-initiator phenyl bis(2,4,6-trimethyl benzoyl) phosphine oxide (BAPO, Ciba Geigy) (300 mg BAPO in 1.5 mL CH₂Cl₂) was added into a pre-dissolved polymer/CH₂Cl₂ solution (1.5 g/500 μ L) and mixed thoroughly. For different experimental purposes, the mixture was transferred into different molds formed by two glass plates and a silicone spacer. Photo-crosslinking of PPF/mPOSS was processed under ultraviolet (UV) light ($\lambda = 315\text{-}380$ nm) from a Spectroline high-intensity long-wave UV lamp (SB-100P, Intensity: 4800 μ w/cm²). The samples used to study the crosslinking kinetics were crosslinked for 0.5, 1, 2, 3, 4, or 5 min. Unless otherwise noted, 5 min was applied to crosslink samples for physical characterizations, degradation, and cell studies. Crosslinked polymer disks and strips were removed from the molds after being cooled to ambient temperature. Then these samples were soaked in acetone for 2 days and dried completely in a vacuum oven at 40 °C. PPF/mPOSS blends were prepared with various mPOSS weight compositions for different characterization methods. For studying crosslinking kinetics, PPF/mPOSS

blends with ϕ_m of 0, 5%, and 10% were prepared. For Fourier-transform infrared (FTIR) measurement and thermogravimetric analysis (TGA), HPPF/mPOSS blends with ϕ_m of 0, 25%, 50%, and 100% were prepared. For Differential Scanning Calorimetric (DSC) measurements, PPF/mPOSS blends with ϕ_m of 0, 5%, 10%, 15%, 25%, 40%, and 100% were prepared. For other physical characterizations and cell studies, PPF/mPOSS blends with ϕ_m of 0, 5%, 10%, and 15% were prepared.

2.2.3 Characterization of Uncrosslinked and Crosslinked PPF/mPOSS Samples

Structural characterization of uncrosslinked PPF/mPOSS blends was performed on a Perkin Elmer Spectrum Spotlight 300 FTIR spectrometer with diamond Attenuated Total Reflectance (ATR). The resolution of the instrument was specified as 4 cm^{-1} at the wavenumber of 1000 cm^{-1} . The gel fraction was measured by immersing polymer disks in excess CH_2Cl_2 for 24 h and calculated using the equations of $W_d/W_0 \times 100\%$, where W_0 is the original weight of disk and W_d is the dry weight of disk after being soaked in CH_2Cl_2 and the subsequent removal of solvent.

DSC tests were performed on a Mettler-Toledo differential scanning calorimeter in a nitrogen atmosphere. To keep the same thermal history, each sample was first heated from room temperature to $100\text{ }^\circ\text{C}$ and cooled to $-90\text{ }^\circ\text{C}$ at a cooling rate of $5\text{ }^\circ\text{C}/\text{min}$. Then a subsequent heating run was performed from -90 to $100\text{ }^\circ\text{C}$ at a heating rate of $10\text{ }^\circ\text{C}/\text{min}$. DSC scans for the last cooling and heating runs were recorded for analysis using the Mettler software. TGA was performed using a Mettler-Toledo Instruments thermogravimetric analyzer in flowing nitrogen at a heating rate of $20\text{ }^\circ\text{C}/\text{min}$.

Tensile measurements were performed by a dynamic mechanical thermal analyzer

(DMTA) at 37 °C. Briefly, crosslinked PPF/mPOSS strips ($\sim 20 \times \sim 1.5 \times \sim 0.3$ mm, length $\times$ width $\times$ thickness) were elongated at a strain rate of 0.001 s^{-1} . Tensile modulus (or Young's modulus) E was determined and averaged from the initial slopes in the stress–strain curves. Stress and strain at break were also obtained from the curves. At least five specimens for each sample were measured and averaged. Linear viscoelastic properties of uncrosslinked samples were determined using a strain-controlled rheometer (RDS-2, Rheometric Scientific) in a frequency range of 0.5-100 rad/s at 25, 37, and 60 °C. Oscillatory shear measurements were performed using a parallel plate flow cell with a diameter of 8 mm and a geometry gap of ~ 0.5 mm.

Hydrolytic degradation of crosslinked PPF/mPOSS was performed in 1 N NaOH solution at 37 °C over 5 weeks. Different disks were used for different degradation time with one week as the interval. After complete drying, the solid residue of a polymer disk was weighed and compared with its initial weight.

2.2.4 Contact Angle and Protein Adsorption

One drop of 10 μL de-ionized water ($\text{pH} = 7.0$) was injected on the surface of crosslinked PPF/mPOSS. After equilibrium reached in 30 s, the water contact angle was measured on a Ramé-Hart NRC C. A. goniometer (model 100-00-230, Mountain Lakes, NJ).

Crosslinked PPF/mPOSS disks (9 mm, diameter) were immersed in α -Minimum Essential Media (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sera-Tech, Germany) and 1% penicillin/streptomycin (Gibco) for 4 h at 37 °C. This medium was for culturing mouse MC3T3 cells. Then the disks were transferred into

48-well plates (one disk per well) and 500 μ L of phosphate buffered saline (PBS) was used to wash the disks three times. Ten minutes of gentle agitation was applied and PBS was discarded after each wash. Three hundred fifty microliters of 1% sodium dodecyl sulfate (SDS) solution was put in these wells for 1 h at room temperature. This step was repeated twice and all the SDS solution was collected in a plastic vial. By using a MicroBCA protein assay kit (Pierce, Rockford, IL), the protein concentration in the collected SDS solution was determined on a microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA) at 562 nm. Albumin in the kit was used to prepare solutions in SDS with eight known concentrations for making a standard calibration curve.

2.2.5 Cell Culture and *In Vitro* Cytocompatibility

Polymer disks were sterilized in excess 70% ethanol solution overnight and fully dried in a vacuum oven for 24 h, and then washed by PBS at least three times prior to use. To evaluate their suitability for bone tissue engineering applications, mouse MC3T3-E1 cells (CRL-2593, ATCC, Manassas, VA) were used in examining cytocompatibility of crosslinked PPF/mPOSS and cell attachment, spreading, and proliferation on the disks. Cells were thawed and plated on polystyrene flasks with cell culture medium described in Section 2.2.4 in an incubator with 5% CO₂ and 95% relative humidity at 37 °C. Cytocompatibility evaluation was carried out by seeding MC3T3 cells in a 24-well plate at a density of 1.8×10^4 cells/cm² with 1 mL of primary medium. Sterile polymer disks placed in trans-wells were immersed in culture medium and exposed to the cells for 1, 2, and 4 days. Empty wells and cell-seeded wells with the same density but no exposure to polymer disks were used as negative and positive controls, respectively. The number of

viable cells was correlated to the UV absorbance at 490 nm determined on the incubated MTS assay solution (CellTiter 96 Aqueous One Solution, Promega, Madison, WI) using the same microplate reader in Section 2.2.4. Cell viability was then evaluated by normalizing the absorption values to the positive control.

2.2.6 *In Vitro* Cell Attachment and Proliferation

Polymer disks were attached on the bottom of 48-well tissue culture plates using sterilized inert silicon-based high-temperature vacuum grease (Dow Corning, Midland, MI) to prevent them from floating in the culture media. MC3T3 cells were seeded onto the polymer disks at a density of 1.8×10^4 cells/cm² and cultured with 400 μ L of culture medium in each well. MTS assay was performed to quantify cell attachment and proliferation, with tissue culture polystyrene (TCPS) used as positive control. After 4 h, 1, 2, and 4 days, culture media were removed from the wells and polymer disks were washed with PBS twice. Attached cells were fixed with 16% paraformaldehyde (PFA) solution for 10 min, washed twice with PBS and permeabilized using 0.2% Triton X-100. Then the cells were stained with rhodamine-phalloidin for 1 h at 37 °C and photographed on an Axiovert 25 Zeiss light microscope (Carl Zeiss, Germany). Cell area was determined and averaged on 20 non-overlapping cells at 4 h post seeding using the ImageJ software (National Institutes of Health, Bethesda, MD).

2.2.7 ALP Activity and Calcium Content

After MC3T3 cells were cultured on crosslinked PPF/mPOSS disks for 7 and 14 days, the alkaline phosphatase (ALP) activity and calcium content were measured using cell lysate prepared using the literature method [149,150]. Cells were washed twice with PBS, trypsinized, and washed again before centrifuge at 1000 rpm for 4 min. The

obtained cell pellet was re-suspended in 1 mL of 0.2% Nonidet P-40 (American Bioanalytical, Natick, MA) and sonicated in an ice bath for 2 min. Cell lysate was frozen at -20 °C before ALP activity and calcium content were measured. ALP activity of cell lysate was measured using fluorescence-based ALP Detection Kit (Sigma, St. Louis, MO). Briefly, 20 µL cell lysate was placed into each well of 96-well plate and incubated at 65 °C for 20 min to minimize the background and phosphatase activities. The majority of the protein products of ALP genes were stable at 65 °C. The samples were cooled to room temperature. Then 20 µL of dilution buffer, 160 µL of fluorescent assay buffer, and 1 µL of the 10 mM substrate solution provided by the kit were added into each well. Then 4 µL of the cell lysate or control enzyme with known concentrations was added to each well and mixed. The absorbance value was measured at 340 nm on the microplate reader. Absolute quantification was achieved using a standard curve constructed with different amounts of control enzyme. Calcium content was determined using QuantiChrom calcium assay kit (BioAssay Systems, Hayward, CA). Briefly, 5 µL cell lysate was transferred into each well of 96-well plate and mixed with 200 µL working reagent. After incubation at room temperature for 3 min, the optical density was measured at 620 nm on the microplate reader. Absolute calcium content was determined using a standard curve constructed with eight known concentrations.

2.2.8 Reverse Transcription (RT) and Real-time Polymerase Chain Reaction (PCR)

MC3T3-E1 cells were seeded on polymer sheets ($\sim 20 \text{ cm}^2$, area) at a density of $\sim 15000 \text{ cells/cm}^2$ and cultured for 14 days. Cells were then trypsinized and total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA). The amount of total RNA

Table 2.1. Oligonucleotide primer sequences for RT and real-time PCR

Gene	Primer sequence	Product Size (bp)
ALP	F: GCCCTCTCCAAGACATATA	372
	R: CCATGATCACGTCGATATCC	
Osteocalcin	F: CAAGTCCCACACAGCAGCTT	371
	R: AAAGCCGAGCTGCCAGAGTT	
GAPDH	F: ACTTTGTCAAGCTCATTTC	267
	R: TGCAGCGAACTTTATTGATG	

from each sample was quantified using Nanodrop1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Reverse transcription of isolated RNA was then performed using DyNAmo cDNA synthesis kit (Thermo Scientific) according to the manufacturer's protocol.

The oligonucleotide primers used for both RT and real-time PCR were listed in Table 2.1. PCR amplification of ALP, osteocalcin, and housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were conducted using a Peltier Thermo Cyclers (PTC-200, MJ Research, Waltham, MA). Thirty PCR cycles for ALP and twenty-seven cycles for osteocalcin and GAPDH were implemented with each cycle consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s. The products were analyzed by electrophoresis using 5 µL samples for 20 min at 100 V in 1.5% agarose gel stained with Gelgreen (Biotium, Hayward, CA). DNA fragments were visualized and imaged using EpiChemi II Darkroom Imaging Systems (UVP, Upland, CA).

Real-time PCR reactions were performed in 25 µL of PCR mixture consisting of each cDNA sample, a specific primer, and a Power SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). Same thermal conditions mentioned above were applied using a Peltier Thermal Cyclers with fluorescence detection systems (PTC-200, MJ Research). Expression of target genes was normalized to the level of GAPDH.

2.2.9 Statistical Analysis

Student's *t*-test was performed to assess the statistical significance ($p < 0.05$) of the difference between results.

2.3 Results and Discussion

2.3.1 Structural Characterization of Uncrosslinked PPF/mPOSS

The chemical composition of uncrosslinked PPF/mPOSS was confirmed and exemplified using the FTIR spectra of HPPF, mPOSS, and their blends with ϕ_m of 25%, 50%, and 75% shown in Figure 2.1. The absorption bands at 1740 and 1635 cm^{-1} in the spectra could be assigned to the ester carbonyl ($\text{C}=\text{O}$) and vinyl ($\text{H}_2\text{C}=\text{CH}-$) groups for all the samples, respectively [36,37]. Consistent with earlier reports [130,132,133,137], the intensities of the characteristic peaks such as C-H stretching of Si-C at $\sim 3000 \text{ cm}^{-1}$ and symmetric stretching of Si-O-Si peak at $\sim 1090 \text{ cm}^{-1}$ demonstrated the dependence on ϕ_m .

2.3.2 Thermal Properties of Uncrosslinked PPF/mPOSS

As shown in the DSC curves in Figure 2.2, T_g values of amorphous HPPF and LPPF were 9.2 and -18.7 $^{\circ}\text{C}$, respectively. This difference in T_g was consistent with the reported strong molecular-weight dependence when the M_n of PPF is lower than 5000 g/mol [24]. Pure amorphous mPOSS had a much lower T_g of -55.6 $^{\circ}\text{C}$ while it had no crystalline/melting peaks up to 100 $^{\circ}\text{C}$. Both PPF and mPOSS were amorphous and transparent at room temperature and soluble in CH_2Cl_2 . For both HPPL/mPOSS and LPPF/mPOSS, one single glass transition was found in their DSC curves when ϕ_m was lower than 15%. In contrast, two glass transitions can be observed when ϕ_m was 15% or

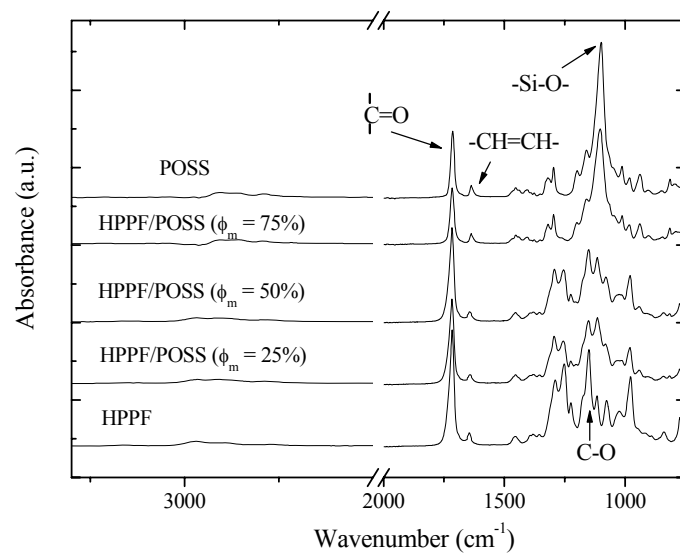


Figure 2.1. FTIR spectra of uncrosslinked HPPF/mPOSS blends.

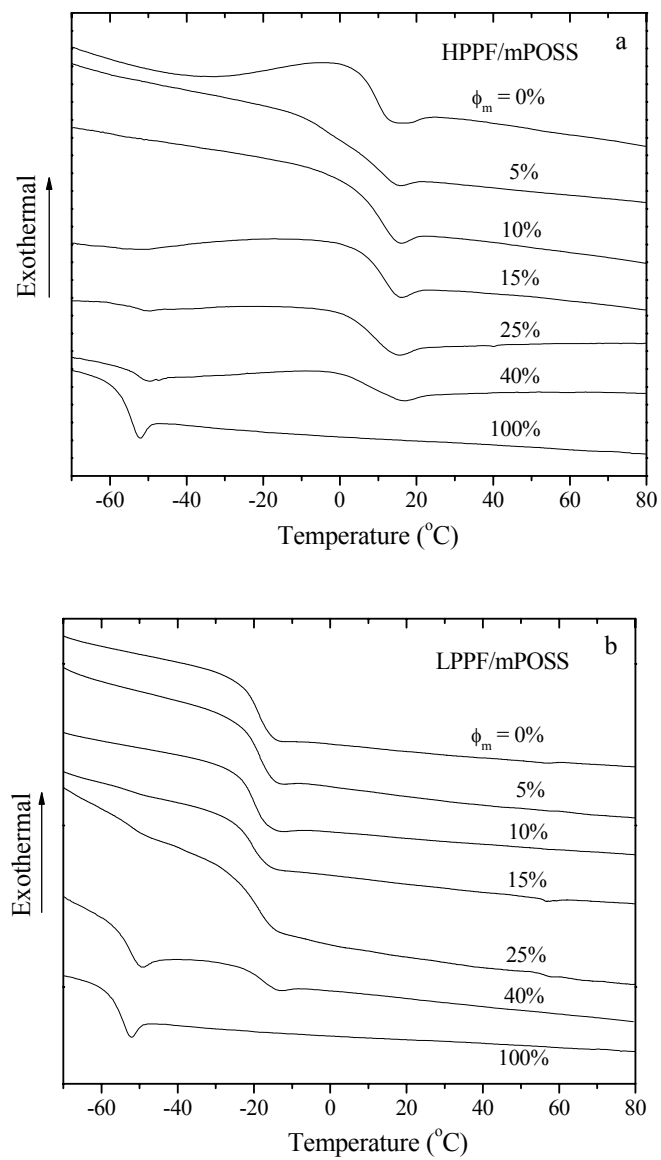


Figure 2.2. DSC curves of uncrosslinked HPPF/mPOSS (a) and LPPF/mPOSS (b) blends.

larger, indicating phase separation occurred in the blends. It was also found that mPOSS could disperse in PMMA up to ϕ_m of 10% before significant phase separation [134]. Unlike the reported plasticization effect of mPOSS on PMMA [134], no evident composition dependence for T_g can be observed from the DSC curves for PPF/mPOSS blends possibly because both HPPF and LPPF had fairly low T_g values.

2.3.3 Viscosity of Uncrosslinked PPF/mPOSS

The zero-shear viscosities (η_0) of uncrosslinked PPF/mPOSS at different temperatures were determined from the Newtonian region where polymer viscosity is independent of frequency. For both uncrosslinked HPPF/mPOSS and LPPF/mPOSS, the composition, temperature, and molecular weight dependence for η_0 can be observed from Figure 2.3a. In agreement with an earlier study [134], η_0 decreased progressively when more mPOSS liquid was introduced into the blends because mPOSS could be considered as a diluent for PPF and thus it could improve the injectability or processability of PPF before the network formation. Lower viscosities could be achieved for uncrosslinked PPF/mPOSS blends as the measurement temperature was raised from 25 to 37 or 60 °C. Moreover, HPPF/mPOSS blends had much higher viscosities than the LPPF/mPOSS blends at the same composition because the mobility of long chains decreased significantly compared with the short chains. As reported earlier, the exponent a in $\eta_0 \sim M^a$ can be as high as 7.0 at 25 °C and it decreases at higher temperatures [24].

At a higher temperature, η_0 decreased and the molecular weight dependence of η_0

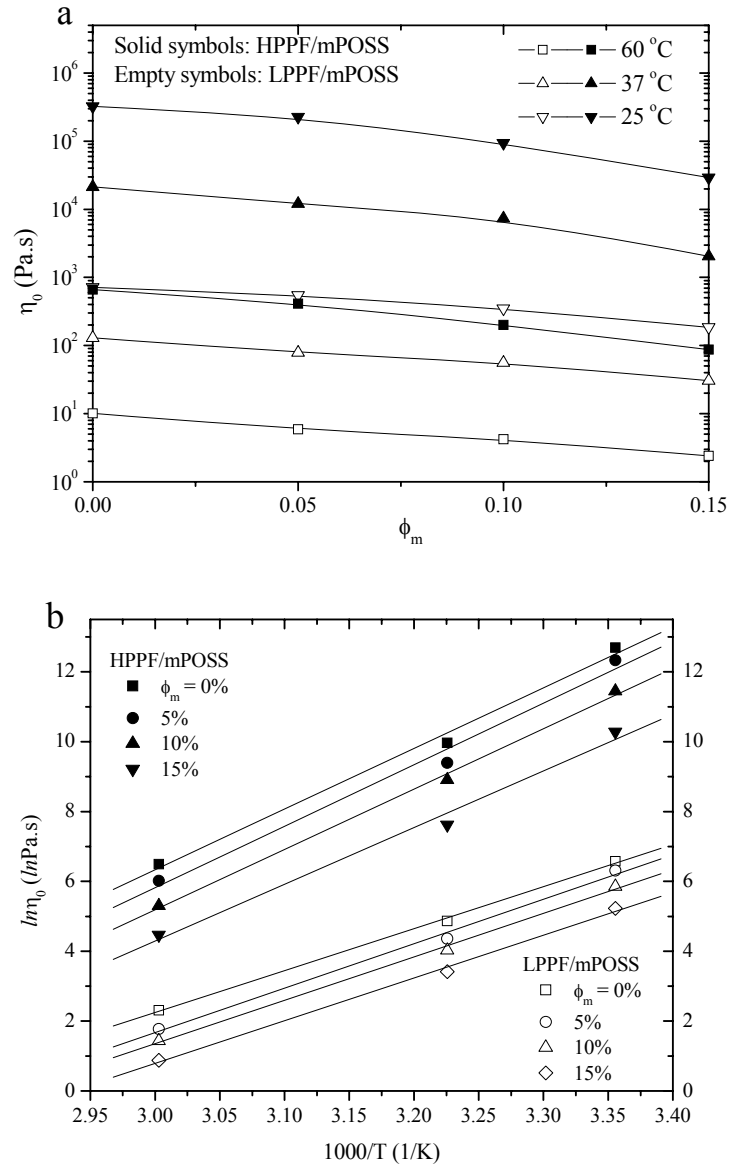


Figure 2.3. (a) Zero-shear viscosities (η_0) of uncrosslinked PPF/mPOSS blends at temperatures of 25, 37, and 60 °C. (b) Temperature dependence of η_0 for uncrosslinked PPF/mPOSS blends with ϕ_m of 0, 5%, 10%, and 15% plotted as $\ln \eta_0$ versus $1000/T$, where T is absolute temperature in K.

was weaker and thus the difference in η_0 between HPPF and LPPF was smaller. The temperature dependence of η_0 can be expressed by using the Arrhenius equation when $\ln \eta_0$ was plotted against $1/T$ in Figure 2.3b. The activation energy for η_0 was obtained from the slope of the curve. The activation energies of 135-147 kJ/mol for the HPPF series were higher than the values of 100-106 kJ/mol for the LPPF series, consistent with an earlier report on the temperature dependence for the viscosity of PPF when its molecular weight was varied [24]. The activation energy showed little variance when different amounts of mPOSS were incorporated with PPF.

2.3.4 Crosslinking Kinetics of PPF/mPOSS

Crosslinking kinetics is important for understanding how a polymer network is formed and when crosslinking is sufficient to ensure sample integrity and suitable mechanical properties while avoid over-cure. As shown in Figure 2.4a, the gel fraction of crosslinked HPPF/mPOSS and LPPF/mPOSS in CH_2Cl_2 both increased asymptotically when the exposure time to UV light was longer. The gel fraction approached to a value higher than 95% for the HPPF series and around 90% for the LPPF series at the crosslinking time of 5 min. At the same crosslinking time, crosslinked HPPF/mPOSS samples demonstrated higher gel fractions than their LPPF/mPOSS counterparts because more covalent double bonds along the HPPF backbone could result in higher crosslinking densities.

The gel fraction for crosslinked PPF/mPOSS varied with ϕ_m and this variance was

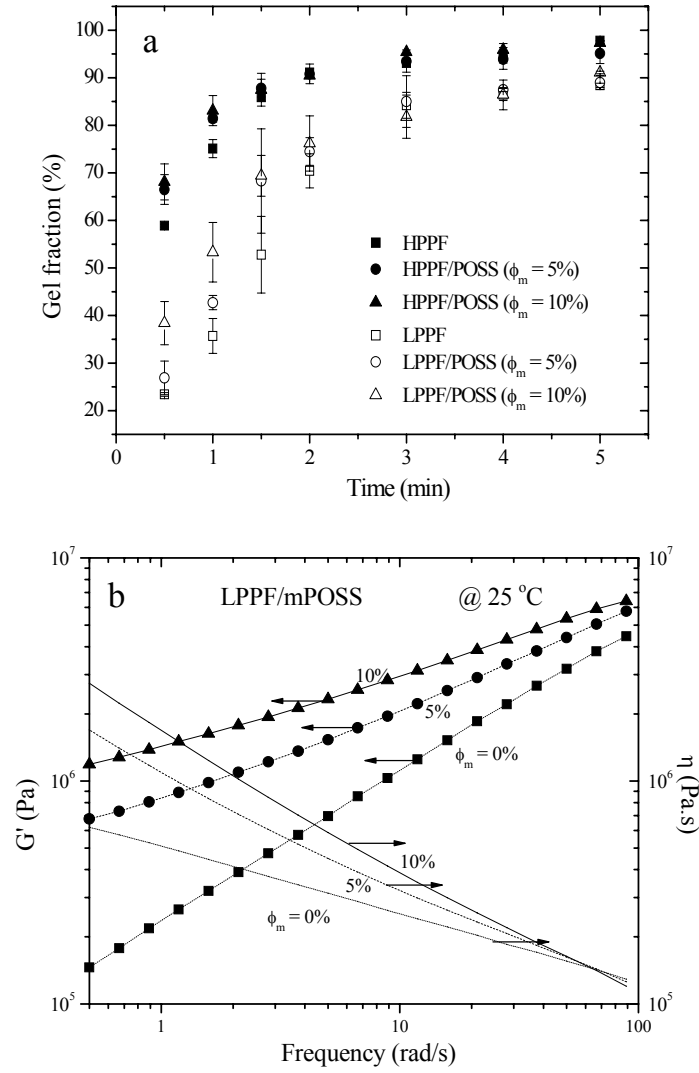


Figure 2.4. (a) Gel fraction of HPPF/mPOSS and LPPF/mPOSS blends after photo-crosslinking for 0.5 to 5 min. (b) Storage modulus G' and viscosity η versus frequency for LPPF/mPOSS blends after photo-crosslinking for 1 min.

more evident in crosslinked LPPF/mPOSS. In the mPOSS compositions of 0, 5%, and 10% studied here, there was only monotonous ϕ_m dependence for the gel fraction. In contrast, crosslinked PPF/DEF samples showed a maximum gel fraction at an intermediate PPF composition of 75% [35]. At the same crosslinking time, the gel fraction of crosslinked PPF/mPOSS increased when ϕ_m was higher, indicating that mPOSS can be an efficient crosslinker because of its eight crosslinkable end groups in one nano-cage. Such enhanced photo-crosslinking efficiency by incorporating mPOSS can also be confirmed from the rheological properties of LPPF/mPOSS samples that were crosslinked for 1 min in Figure 2.4b. Both storage modulus G' and viscosity η as functions of frequency increased upon incorporating more mPOSS into crosslinked LPPF/mPOSS. Once a perfect network is formed, G' will be independent of frequency and η will be approximately inversely proportional to frequency [36]. The results in Figure 2.4b indicates that crosslinked LPPF/mPOSS with higher ϕ_m could have a higher crosslinking density at the same UV exposure time, which is beneficial in fabricating polymeric scaffolds.

SEM images of crosslinked LPPF and LPPF/mPOSS are demonstrated in Figure 2.5. These two samples were exposed to UV light for 3 min on a metal surface with micron-scale features and then washed in acetone to remove the sol fraction. As indicated by good replication of the surface features, it is evident that the integrity of photo-crosslinked LPPF/mPOSS ($\phi_m = 10\%$) was significantly better than that of

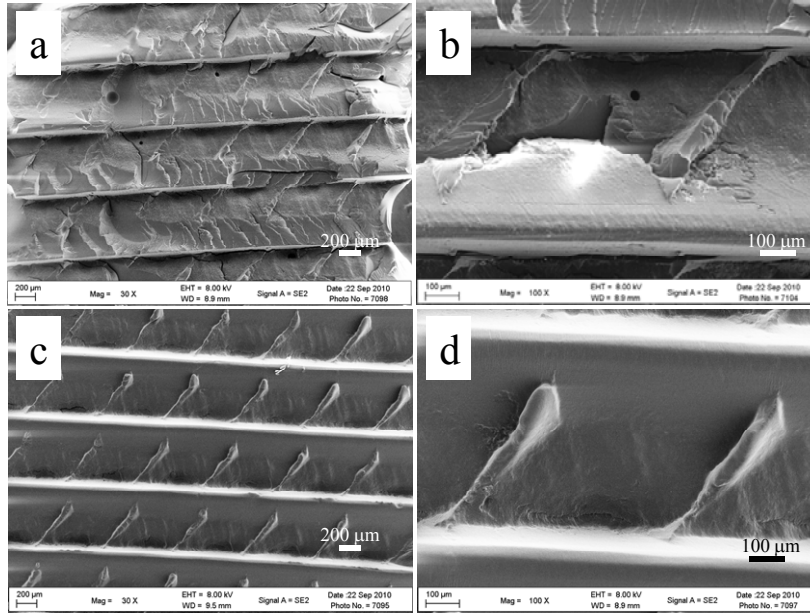


Figure 2.5. SEM images of photo-crosslinked (crosslinking time: 3 min) LPPF (a, b) and LPPF/mPOSS with ϕ_m of 10% (c, d) with surface features at the micron scale. The magnification is $\times 30$ for (a) and (c) and $\times 100$ for (b) and (d). The scale bars represent 200 μm in (a) and (c) while 100 μm in (b) and (d).



Figure 2.6. Transparent disks of photo-crosslinked PPF and PPF/mPOSS samples. Upper row: HPPF/mPOSS; lower row: LPPF/mPOSS; ϕ_m of 0, 5%, 10%, and 15% from left to right.

photo-crosslinked LPPF because it had a higher gel fraction, in agreement with the gel fraction results in Figure 2.4a. Photo-crosslinked PPF/mPOSS disks with ϕ_m from 5% to 15% were as transparent as crosslinked PPF, as demonstrated in Figure 2.6. When ϕ_m was higher than 15%, significant phase separation occurred for crosslinked PPF/mPOSS and these disks were no longer transparent. Similar findings have been reported for crosslinked mPOSS and mPOSS/PMMA [135,137]. Because of POSS aggregates, a multi-layer structure could be observed using Transmission Electron Microscopy (TEM) in clear thermally-polymerized mPOSS and its black blends with a trifunctional furan compound [137,138].

2.3.5 Thermal Stability of Crosslinked PPF/mPOSS

Crosslinked PPF/mPOSS disks prepared using a UV exposure time of 5 min were rigid, strong, and solvent resistant and did not soften or flow at higher temperature. Because segmental motions were limited by the crosslinks, these disks did not show discernible glass transition in DSC curves (not shown), similar to crosslinked PPF with a high crosslinking density [27]. TGA was performed to determine thermal stability of crosslinked PPF/mPOSS by measuring the weight loss when the temperature was raised from room temperature to 700 °C. As demonstrated in Figure 2.7, all the crosslinked samples showed one single major degradation step at the onset thermal degradation temperature T_d . However, when derivative of weight change was plotted against temperature in Figures 2.7c and 2.7d, only crosslinked PPF had one single peak at ~380

°C while multiple peaks with intensities proportional to the composition of the ascribed component were observed in the mPOSS-containing samples. The two peaks at ~440 and ~500 °C in Figures 2.7c and 2.7d can be attributed to release of CO₂ and H₂O from oxidation of –Si-CH₂– groups in mPOSS, respectively [132,133]. The peak temperatures all shifted to higher values with increasing ϕ_m .

T_d demonstrated both molecular weight and composition dependence. When ϕ_m in crosslinked PPF/mPOSS increased from 0 to 25%, 50%, and 75%, T_d increased progressively from 350 to 352, 360, and 383 °C for crosslinked HPPF/mPOSS, and from 342 to 344, 351, and 380 °C for crosslinked LPPF/mPOSS, respectively. The slight difference in T_d between two crosslinked PPF samples was in agreement with the previous finding that T_d for PPF increased with molecular weight from ~320 to ~350 °C [24]. Pure crosslinked mPOSS had a higher T_d of 415 °C. mPOSS participated the network can restrict the thermal motions of the polymer chains effectively and can also provide additional heat capacity thereby stabilizing the materials against thermal decomposition [130]. The present result was also consistent with enhanced thermal stability of the polymers by incorporating mPOSS to methysilicone resin [132,133]. Meanwhile, the residue fraction was 7.5% for crosslinked HPPF and 8.1% for crosslinked LPPF. It increased proportionally with ϕ_m up to a value of 46.4% for crosslinked mPOSS. The residue was silica as suggested using FTIR spectrum in an earlier report [132,133]. Based on ϕ_m and the residue fractions for two pure components, the predicted values for

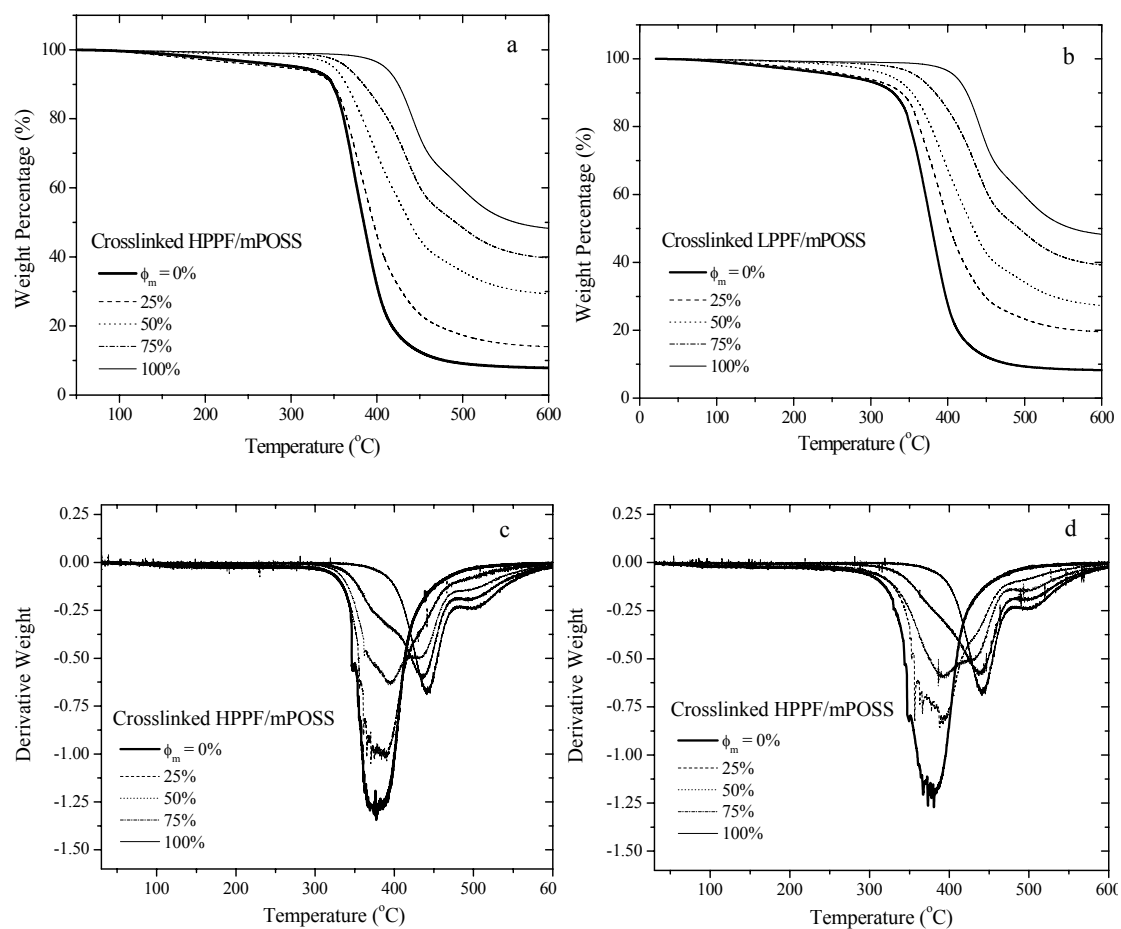


Figure 2.7. TGA thermograms (a, b) and derivative thermogravimetric (DTG) curves (c, d) of crosslinked HPPF/mPOSS (a, c) and LPPF/mPOSS (b, d).

crosslinked PPF/mPOSS agreed with the TGA data within experimental errors. The high residue fractions of the crosslinked samples with mPOSS indicated their high flame retardation.

2.3.6 Mechanical Properties of Crosslinked PPF/mPOSS

The mechanical properties of crosslinked PPF/mPOSS determined from tensile testing at physiological temperature are shown in Figure 2.8. The representative stress-strain curves in Figure 2.8a clearly indicated that crosslinked LPPF/mPOSS samples at all compositions were more compliant than their HPPF/mPOSS counterparts because of their lower crosslinking densities. This trend was also reported in comparing crosslinked HPPF/DEF samples with crosslinked LPPF/DEF ones [35].

As shown in the inset of Figure 2.8a, E increased progressively from 1701 ± 51 MPa for crosslinked PPF to 1975 ± 86 MPa for crosslinked HPPF/mPOSS ($\phi_m = 15\%$). The experimental values of E were consistent with previous reports on crosslinked PPF [36,38]. For crosslinked LPPF/mPOSS, E first increased from 330 ± 33 to 516 ± 66 MPa when ϕ_m increased from 0 to 10% and then decreased slightly to 495 ± 75 MPa for ϕ_m of 15%, possibly due to phase separation. The results demonstrated that E can be enhanced because of the nanoscale reinforcement in the polymer network from the POSS cage. As shown in Figure 2.8b, the stress at break of crosslinked HPPF/mPOSS increased from 32.7 ± 0.7 to 36.2 ± 0.9 MPa while the strain at break decreased from $2.9 \pm 0.3\%$ to $2.2 \pm 0.2\%$ when ϕ_m increased from 0 to 15%. Correspondingly, stress at break increased

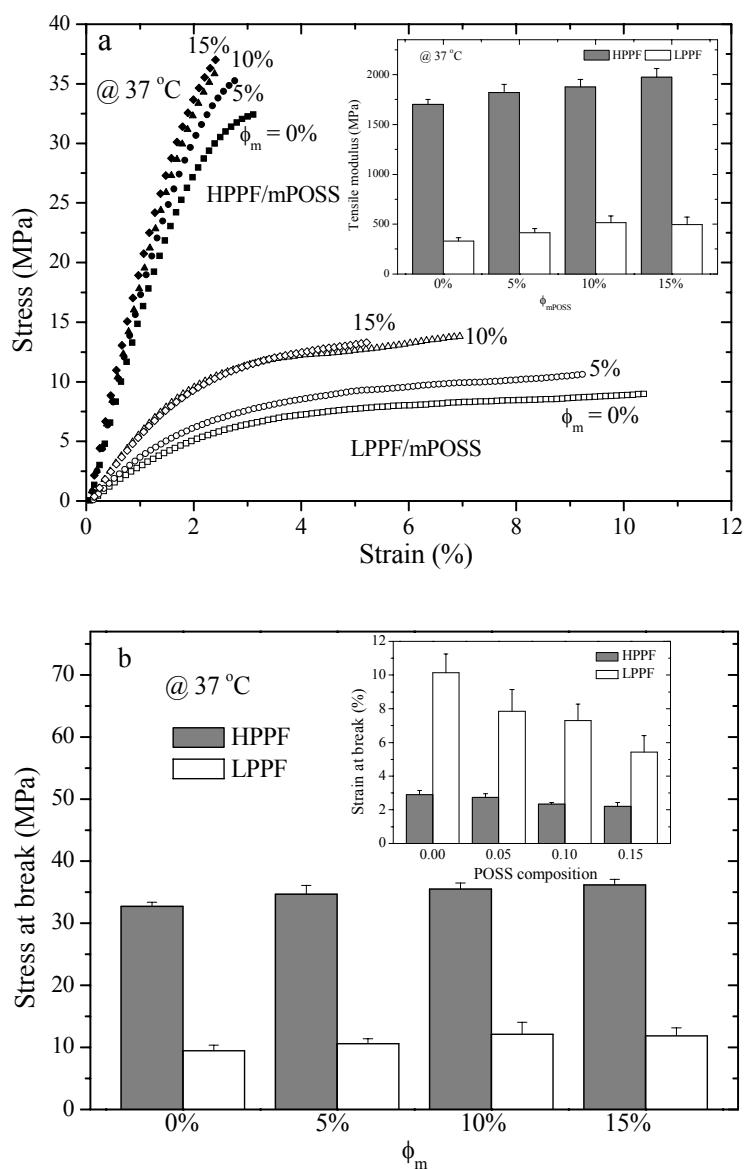


Figure 2.8. (a) Representative stress-strain curves from tensile testing and tensile moduli (inset) of crosslinked PPF/mPOSS at 37 °C. (b) Stress and strain (inset) at break of crosslinked PPF/mPOSS at 37 °C.

slightly from 9.5 ± 0.9 to ~ 12.0 MPa while strain at break decreased from $10.1 \pm 1.1\%$ to $5.4 \pm 1.0\%$ for crosslinked LPPF/mPOSS. The change in mechanical properties was monotonous in the composition range studied here. Higher ϕ_m may result in less desirable mechanical properties because of phase separation, lower double bond conversion, and slower photo-crosslinking rate, as found in photo-crosslinked blends of mPOSS and 2,2'-bis-[4-(methacryloxypropoxy)-phenyl]-propane (Bis-GMA) [143]. Non-linear dependence of tensile modulus on the composition of DEF was also reported when HPPF or LPPF was crosslinked with DEF, which could increase the elastic modulus of crosslinked PPF in the DEF composition range of 0-25% [35].

2.3.7 Degradation of Crosslinked PPF/mPOSS in NaOH Solution

Similar to photo-crosslinked PPF [21,26,36], no detectable degradation could be observed in one week for all crosslinked PPF/mPOSS samples in PBS at 37 °C. The time needed to *in vitro* degrade 50% of the original weight of crosslinked PPF can be as long as 52 weeks [21,26]. Consistent with other POSS-containing polymer composites [126,144], the biostability of crosslinked PPF/mPOSS is advantageous for bone tissue engineering applications, which require sustainable mechanical strength before new bone tissue ingrowth. To demonstrate degradability, the weight loss of crosslinked PPF/mPOSS at different exposure time in accelerated hydrolytic degradation in 1 N NaOH aqueous solution at 37 °C has been studied and shown in Figure 2.9. The weight loss increased continuously with the exposure time for all the samples. After the first week, the

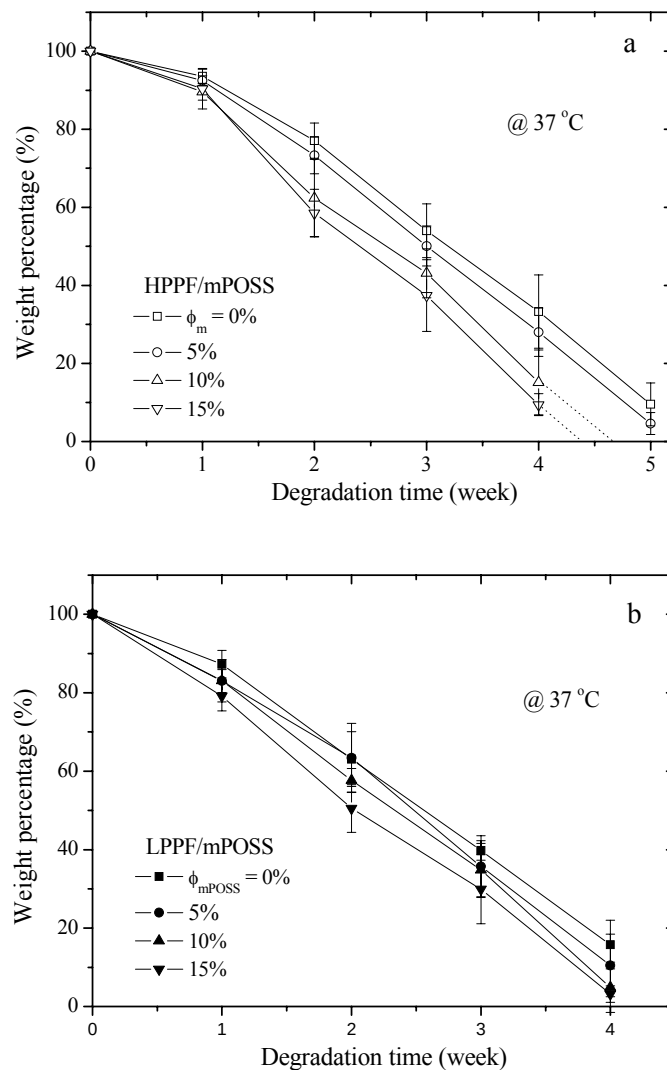


Figure 2.9. Weight loss of crosslinked HPPF/mPOSS (a) and LPPF/mPOSS (b) samples at different degradation times in 1 N NaOH aqueous solution at 37 °C. The dotted lines in (a) were drawn following the earlier trend to indicate crosslinked HPPF/mPOSS disappeared between 4 and 5 weeks.

degradation rate indicated by the slope of the weight loss curve was almost a constant over time.

At given exposure time, the weight loss was higher by incorporating more mPOSS into crosslinked PPF/mPOSS. Blending mPOSS could slightly accelerate the hydrolytic degradation of crosslinked PPF/mPOSS in both series. Besides cleaving the ester bonds in crosslinked PPF/mPOSS, NaOH could react rapidly with uncrosslinked POSS frameworks and open the cage to silanol end groups [151], though solvent-resistant crosslinked disks significantly hindered this reaction. We also immersed photo-crosslinked mPOSS in a 10 N NaOH aqueous solution at 80 °C and found it could completely break into small brown pieces in 24 h. It was reported earlier that POSS could more significantly accelerate the hydrolytic degradation of poly(L-lactide) (PLLA) in the nanocomposites with respect to neat PLLA in NaOH solution (pH = 13); however, the exact mechanism was unknown and can be very different from the case here [152]. As mentioned earlier about biostability, POSS nanocores could shield the soft segments of the polyurethane from degradation in solutions other than NaOH aqueous solution [144]. Crosslinked LPPF/mPOSS disks with weaker mechanical strengths degraded faster than crosslinked HPPF/mPOSS disks, in good agreement with the earlier finding that the degradation rate of crosslinked PPF and PPF-diacrylate increases with decreasing crosslinking density because fewer chains need to be cleaved to disrupt the networks [21,26]. Crosslinked HPPF/mPOSS disks with ϕ_m of 10% or 15% disappeared at week 5.

From the trend prior to week 5, it can be concluded that these samples degraded completely between 4 and 5 weeks, indicated by the dotted lines in Figure 2.9a. As implied in Figure 2.9b, crosslinked LPPF/mPOSS samples could disappear earlier within 5 weeks in 1 N NaOH solution at 37 °C.

2.3.8 Surface Hydrophilicity and Protein Adsorption

All the crosslinked PPF/mPOSS disks prepared between two smooth glass plates had similar surface morphology because of high gel fractions, as demonstrated in Figure 2.10. The root-mean-square (rms) surface roughnesses determined from the AFM images were close to each other among all the samples. Surface hydrophilicity and the ability to adsorb serum proteins from culture media are critical to cell attachment and proliferation. As an indicator of surface energy and wettability, the water contact angle was $83.2 \pm 0.7^\circ$ for crosslinked HPPF and $77.5 \pm 0.9^\circ$ for crosslinked LPPF. Crosslinked PPF became more hydrophobic when the molecular weight was higher because the network had a higher crosslinking density and a lower density of chain ends. By blending 15% mPOSS, the water contact angle only slightly increased by $\sim 2^\circ$ for crosslinked PPF/mPOSS, suggesting that the surface wettability was not affected significantly after incorporating mPOSS. Measured by using the MicroBCA kit, the amount of proteins adsorbed on the disk surface showed little variance over all crosslinked PPF/mPOSS samples, as indicated in Figure 2.11. No evident roles of PPF molecular weight, hydrophilicity, or ϕ_m in determining protein adsorption on polymer surfaces could be found from the present

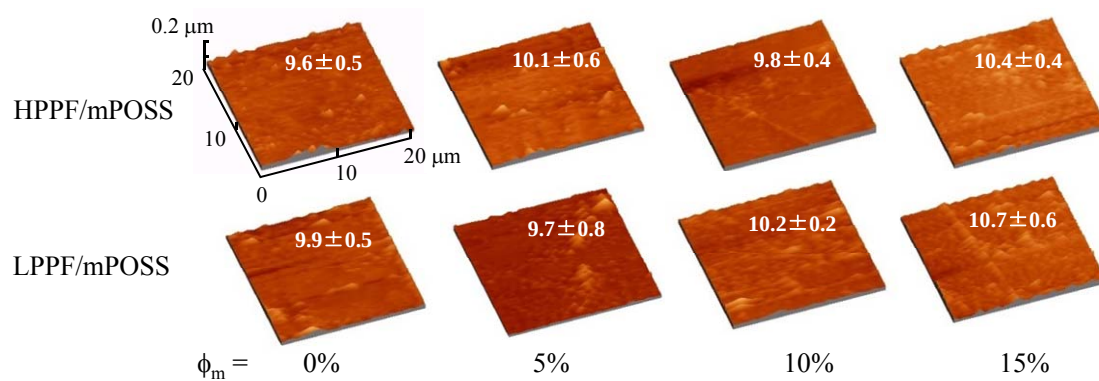


Figure 2.10. AFM images of crosslinked PPF/mPOSS disks. Surface roughness is represented by the number in nm with standard deviation in each image.

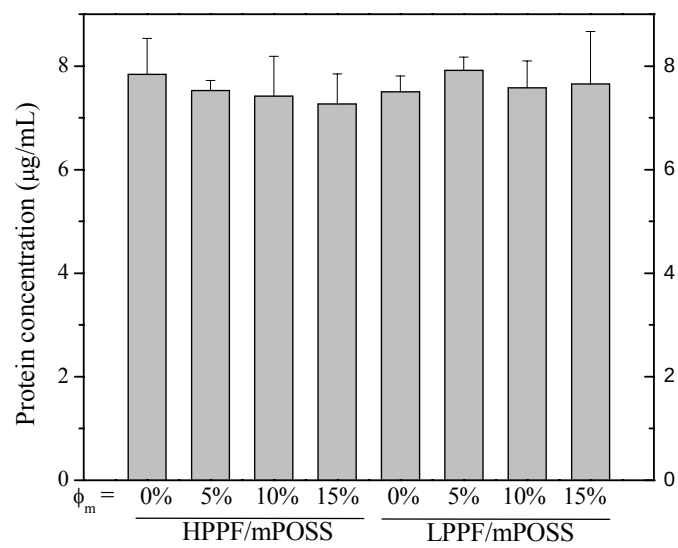


Figure 2.11. Protein adsorption on crosslinked PPF/mPOSS disks.

results.

2.3.9 Cell Viability, Attachment, Spreading, and Proliferation

As shown in Figure 2.12, the fractions of viable MC3T3 cells cultured in the presence of crosslinked PPF/mPOSS disks normalized to the positive control were close to 100% at days 1, 2, and 4 post-seeding. Similar to earlier studies on crosslinked PPF using rat bone marrow stromal cells [36,153], the present result indicated that no leachable toxic molecules were released from the samples and all the samples had excellent compatibility to MC3T3 cells in the period for evaluating cell attachment, spreading, and proliferation. An earlier study using primary human gingival fibroblasts and mouse fibroblast cells demonstrated that mPOSS could even improve the biocompatibility of acrylic-based dental materials by showing a lower mutagenicity and similar cell metabolic activity in the 72-h immersion when POSS-containing hybrid composites were compared with the positive control group [142].

Cell attachment and spreading on polymer substrates are critical for cell motility, growth, and tissue organization when polymers are considered for tissue engineering applications. Fluorescent images of attached MC3T3 cells stained by rhodamine-phalloidin are shown in Figure 2.13a. All crosslinked PPF/mPOSS samples could well support cell adhesion that was monitored at 4 h post-seeding and proliferation indicated by the increment in cell number over days 1, 2, and 4. Compared with crosslinked LPPF/mPOSS disks, crosslinked HPPF/mPOSS disks showed more attached

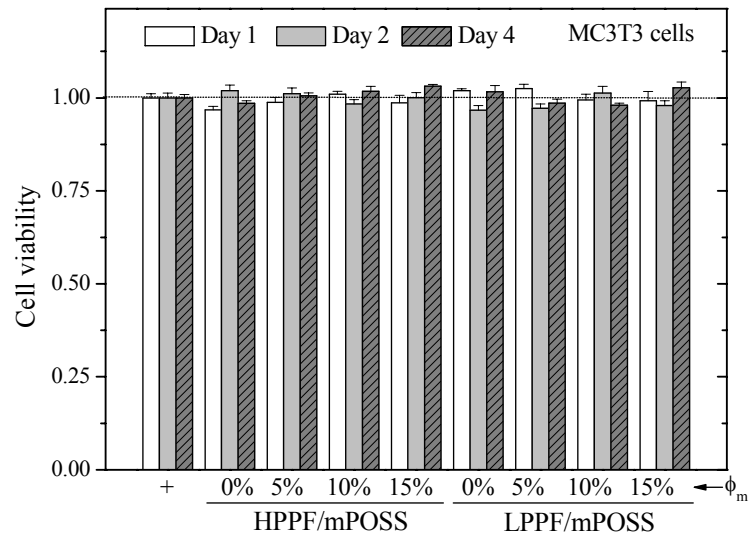


Figure 2.12. Mouse MC3T3-E1 cell viability in the presence of crosslinked PPF/mPOSS compared with TCPS as positive (+) control.

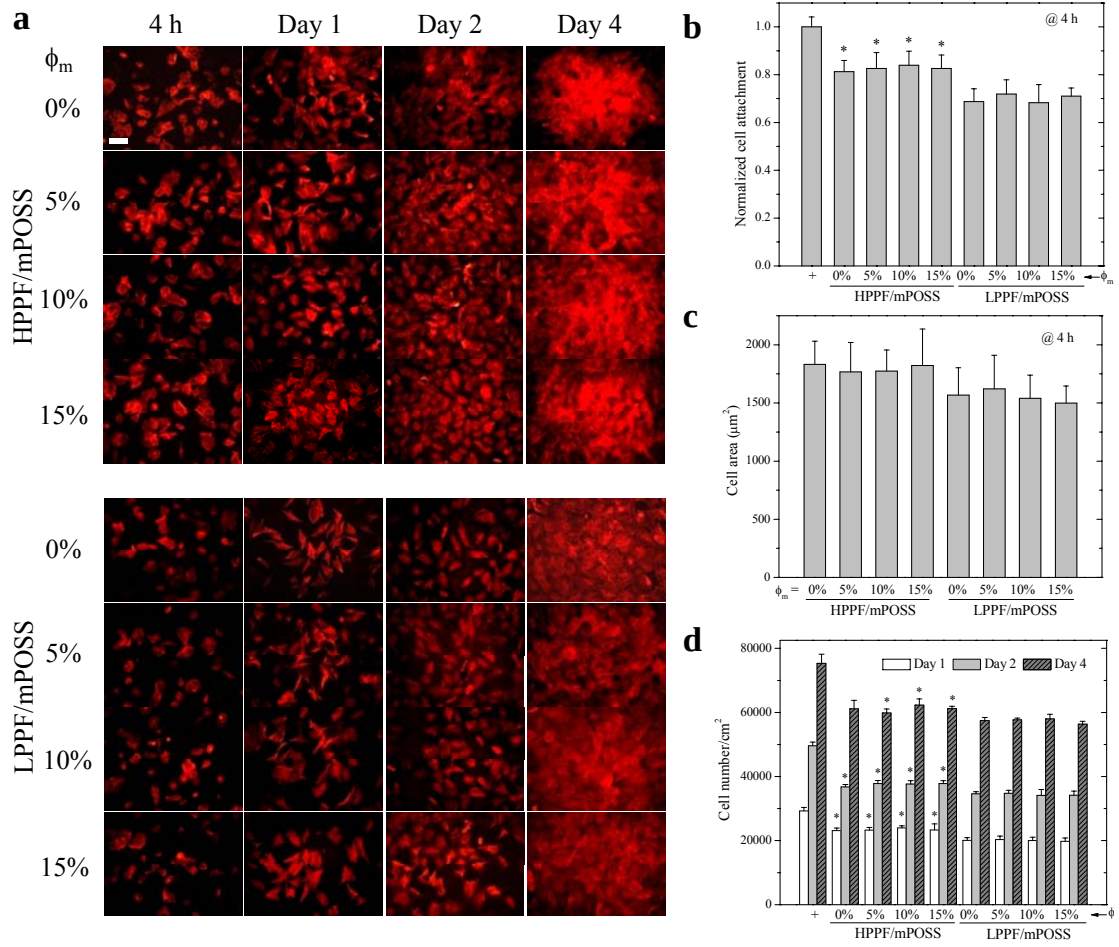


Figure 2.13. (a) Fluorescent images (× 200) of MC3T3 cells stained using rhodamine-phalloidin on crosslinked PPF/mPOSS disks at 4 h, days 1, 2, and 4 post-seeding. The scale bar of 100 μm is applicable to all. (b) Normalized cell attachment at 4 h. (c) Cell average area at 4 h. (d) Cell numbers on crosslinked disks at days 1, 2, and 4, compared to cell-seeded TCPS as positive (+) control. *, $p < 0.05$ relative to TCPS and its crosslinked LPPF/mPOSS counterpart with the same ϕ_m at the same culture time.

cells and these cells had a more spread-out phenotype and a relatively larger average cell area at 4 h. Figure 2.13b shows cell attachment at 4 h post-seeding on crosslinked PPF/mPOSS disks determined by normalizing the MTS values to the value of positive control. It can be seen that normalized MC3T3 cell attachment was higher on crosslinked HPPF than that on crosslinked LPPF but showed little difference when ϕ_m was varied for both series of crosslinked PPF/mPOSS. In addition, the average areas of attached cells at 4 h (Figure 2.13c) were 1830 ± 200 and $1570 \pm 240 \mu\text{m}^2$ for crosslinked HPPF and LPPF, respectively. Represented by the cell numbers measured using MTS assay, cell proliferation over days 1, 2, and 4 showed a trend similar to that in cell attachment in Figure 2.13d. Cell numbers directly counted from the cell images were in good agreement with the data from MTS measurements and demonstrated the same trend when the sample varied. Further discussion on cell-material interactions is elaborated in Section 2.4.

2.3.10 ALP Activity and Calcium Content

MC3T3 cell differentiation on crosslinked PPF/mPOSS substrates was characterized by two important indicators at the onset of osteoblastic differentiation, ALP activity as an early phase marker and calcium content for quantifying mineralization of the extracellular matrix by differentiated cells after culturing for 7 and 14 days [38,149,150]. In general, both ALP activity and calcium content for both 7 and 14 days (Figure 2.14) showed significant differences ($p < 0.05$) between crosslinked HPPF/mPOSS and LPPF/mPOSS, indicating that the molecular weight of PPF can be an important factor in regulating *in*

vitro MC3T3 cell differentiation, possibly via distinct surface stiffnesses of the crosslinked disks. Meanwhile, little difference could be observed between crosslinked PPF and PPF/mPOSS samples with various ϕ_m , except that crosslinked LPPF was significantly lower in calcium content than the samples crosslinked with mPOSS. It should also be noted that ALP activity decreased by a factor of ~40% while the calcium content increased by a factor of ~130% when the cell culture time was prolonged from 7 to 14 days. As an early bone marker, ALP activity has been found to maximize at around one week [154,155]. In contrast, mineralization increases with time according to the relationship between proliferation and differentiation during the rat osteoblast developmental sequence [156].

2.3.11 Osteocalcin and ALP Gene Expression

As demonstrated in Figure 2.15, the expression of osteocalcin and ALP gene transcripts was evaluated by both real-time and RT PCR using total RNA extracted from MC3T3 cells cultured on crosslinked PPF/mPOSS disks. It is evident that both osteocalcin and ALP gene expression levels of MC3T3 cells cultured on crosslinked HPPF/mPOSS were significantly higher than those on crosslinked LPPS/mPOSS with the same ϕ_m . No significant difference could be observed among the same series with four compositions. The roles of PPF molecular weight and ϕ_m in regulating MC3T3 gene expression were consistent with the cell behavior discussed earlier. Both real-time and RT PCR analysis demonstrated that osteocalcin gene expression normalized by GAPDH

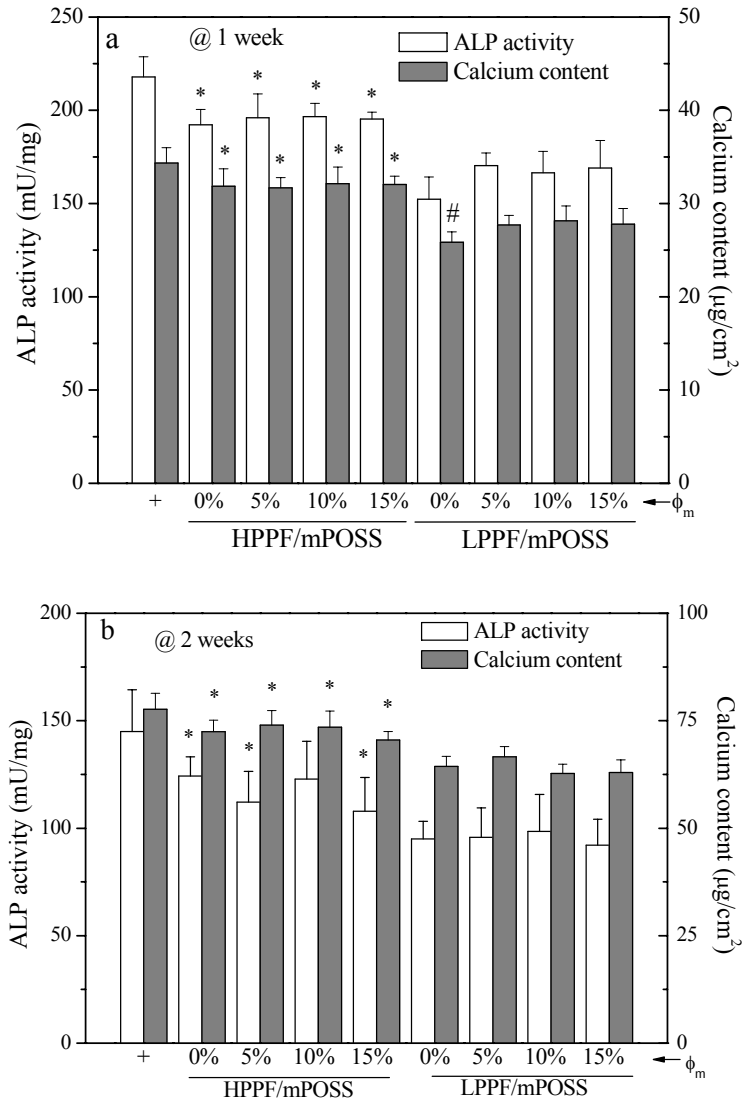


Figure 2.14. ALP activity and calcium content of MC3T3 cells cultured on crosslinked PPF/POSS disks for (a) 7 and (b) 14 days, compared to cell-seeded TCPS as positive (+) control. *, $p < 0.05$ relative to crosslinked LPPF/mPOSS with the same ϕ_m . #, $p < 0.05$ relative to crosslinked LPPF/mPOSS with other ϕ_m at day 7.

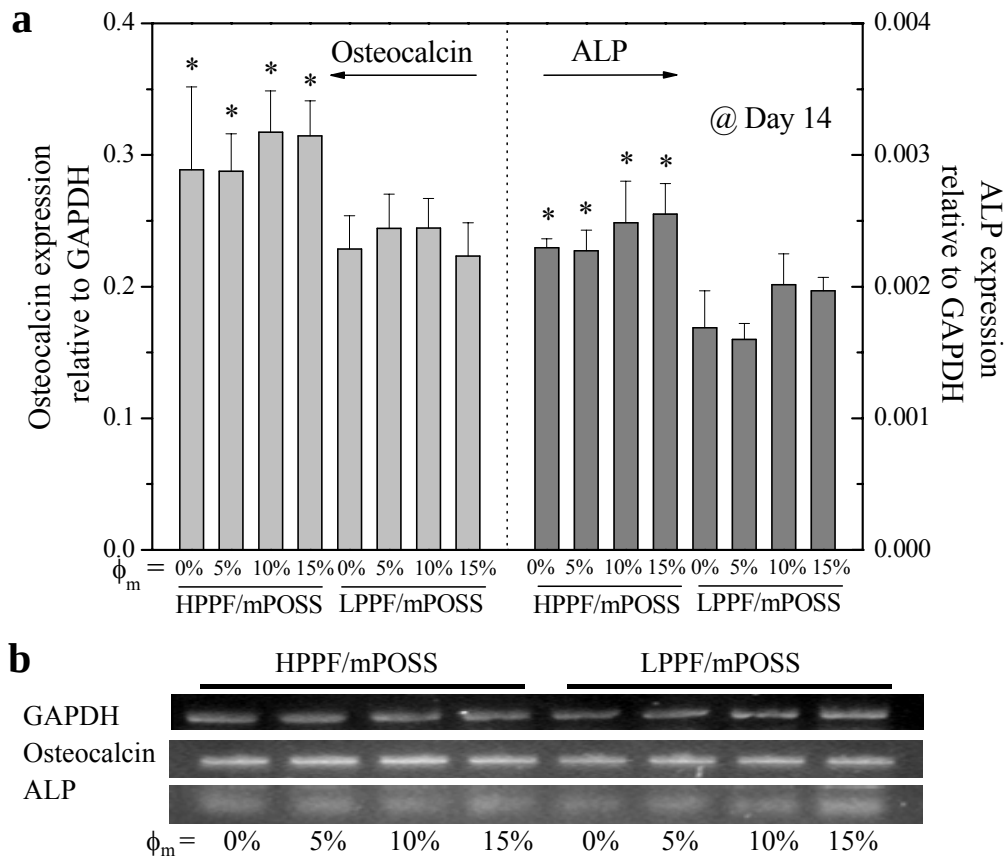


Figure 2.15. Osteocalcin and ALP gene expression normalized by GAPDH in MC3T3 cells cultured on crosslinked PPF/POSS disks for 14 days by (a) real-time and (b) RT PCR analysis. *, $p < 0.05$ relative to crosslinked LPPF/mPOSS with the same ϕ_m .

gene was much higher than ALP gene expression at day 14 because osteocalcin is one of the mature bone-specific markers for osteoblastic differentiation during mineralization and is found in fully developed mineralized matrix while ALP gene follows immediately the proliferative period [154-156].

2.4 Further Discussion

In this study, the role of mPOSS was to expedite the crosslinking and enhance the tensile modulus of crosslinked LPPF and HPPF. All PPF/mPOSS blends demonstrated improved processability as their viscosities were lower than pure PPF. Meanwhile, more efficient photo-crosslinking can be achieved upon exposure to UV light for only 3 min. These results suggest mPOSS can be used as an efficient crosslinker in fabricating PPF scaffolds through stereolithographic methods. Without showing detectable cytotoxicity and significant difference from crosslinked PPF, PPF networks incorporated with mPOSS could support mouse MC3T3 cells attachment, spreading, proliferation, and differentiation. These results indicate that addition of mPOSS will not change MC3T3 cell responses to substrates and scaffolds made from crosslinked PPF. An additional interesting result is that the molecular weight of PPF can result in different physical properties of crosslinked PPF and consequently distinct cell behavior and gene expression.

There are three major categories of surface characteristics to influence cell-material interactions [6-8]. The category of surface chemical factors includes hydrophilicity, capability of adsorbing proteins, functional groups or grafted chains, and characteristics and density of charges [6-8]. Although the surface composition of crosslinked PPF/mPOSS varied with ϕ_m , two important surface chemical factors, hydrophilicity and capability of adsorbing proteins, were almost identical. Thus there was no significant role of ϕ_m in determining cell-material interactions and the discussion is only focused on the properties induced by different molecular weights of PPF. Despite that the HPPF series were more hydrophobic than the LPPF series, the difference of 5.7° in the water contact angle did not result in any difference in the amount of adsorbed proteins or can be used to interpret the significant difference in cell behavior. The reason is generally a more hydrophobic surface with a water contact angle of 83.2° is believed to be less favorable for cell adhesion and proliferation compared to a surface with an intermediate water contact angle between 45° and 75° [6-8]. Surface morphology is also important for regulating cell behavior because both micron- and nanometer-scale surface roughnesses are comparable to the dimensions of cell body, integrin clusters, and individual proteins [6]. In the present study, morphological factors can be ruled out because all these crosslinked disks had good integrity and gel fractions close to 100%. All the crosslinked samples were amorphous without the need to consider surface roughness induced in crystallization.

Mechanical cues such as substrate stiffness have been recognized as one group of important factors in regulating cell behavior and this regulation can be distinct for different cell types [11,12]. The role of surface stiffness has been extensively studied using hydrogel substrates, which normally have surface stiffness less than 1 MPa. A recent combinatorial study on materials properties such as wettability, surface topography, surface chemistry and indentation indicates that the role of indentation elastic modulus can be found in colony formation of human embryonic stem cells when the value was even as high as 1 GPa, although the other factors such as the extent of polymer hydrophobicity-hydrophilicity are intertwined with the mechanical factor [157]. In our previous study, crosslinked PPF/PCLDA had a wide range of E from 6.0 MPa to 1.44 GPa and demonstrated continuous growth in mouse MC3T3 cell proliferation [39]. Compared with their amorphous counterparts with E on the order of 1 MPa, PCLF or PCLDA networks strengthened by crystalline domains had E values around 100 MPa and also demonstrated better bone marrow stromal or MC3T3 cell adhesion and proliferation [56,57,83]. In this study, E only increased by 16.1% for the HPPF series and 56.4% for the LPPF series when 15% mPOSS was added. In comparison, the E value of crosslinked HPPF was more than 5 times as much as that of crosslinked LPPF. Thus we speculate that MC3T3 cells could sense the difference in the PPF molecular weight but not the factor of ϕ_m . The *in vitro* cell results in this study suggest that surface mechanics can be a critical origin for the difference in cell attachment, spreading, proliferation, differentiation, and

gene expression. Further confirmation that the high range of substrate stiffness could induce different cell behaviors is under investigation in our research group.

As an excellent candidate material for bone tissue engineering applications, the molecular weight of PPF can directly determine the mechanical properties of the scaffolds made from crosslinking PPF or PPF/DEF and consequently bone cell function and tissue ingrowth. As found in this study, significantly better MC3T3 cell attachment, proliferation, ALP activity, calcium content, and upregulation of both osteocalcin and ALP genes can be achieved on substrates made from PPF with a higher molecular weight. However, it is difficult to increase the molecular weight of PPF in polycondensation because a longer reaction time itself or incorporated with a higher reaction temperature can induce thermal crosslinking of PPF [23-24], although these two methods are efficient in synthesizing high-molecular-weight saturated polyesters. Nevertheless, a higher molecular weight for PPF may not be desirable because it will result in an exponentially increased viscosity at room temperature that will consequently be detrimental to the processability of PPF [24]. Uncrosslinked PPF may not be used in loading purposes because of its low T_g for infinite molecular weight (31.9 °C) and unentangled characteristics [24]. On the other hand, after crosslinking, PPF with a molecular weight higher than 3000 g/mol may not have a better mechanical strength for promoting bone cell functions.

We are currently applying a novel deposition-and-cure method to manufacture substrates and scaffolds with structural features using photo-crosslinkable or thermoplastic biodegradable polymers. In the presence of mPOSS, PPF can be crosslinked more efficiently at the same UV exposure time into 2D substrates and 3D scaffolds with better precision and controllability, which can be used to study cell/tissue-material interactions. PPF used in this study can have high gel fraction and crosslinking density because it has ~20 (for HPPF) or ~6 (for LPPF) double bonds along one backbone. Thus the addition of mPOSS only increased the tensile modulus and strength slightly. For other photo-crosslinkable polymers such as PCLF and PCLDA [49,83,158], their low crosslinking densities and compliant characteristics can be altered greatly by adding mPOSS and consequently cell behavior on these substrates will differ significantly. Despite its hybrid inorganic-organic nature, mPOSS used in this study was still liquid-like. Similar to previous studies [123-136,141,144-147], solid-like POSS with reactive segments are being used to prepare nanocomposites with PPF and other unsaturated polyesters in our group. Distinct rheological properties of uncrosslinked blends and surface characteristics of crosslinked composites are expected to demonstrate because of the crystalline structure of POSS and enhancement from the solid particles. In particular, polymer networks will be possibly toughened by solid-state POSS and this is advantageous for their biomedical applications such as bone and dental repair.

2.5 Conclusions

We have incorporated methacryl-polyhedral oligomeric silsesquioxane (mPOSS) with poly(propylene fumarate) (PPF) to prepare novel injectable and photo-crosslinkable biomaterials for bone tissue engineering applications. We have investigated the crosslinking kinetics of PPF/mPOSS for two molecular weights of PPF and various compositions of mPOSS. Structural and physical characterizations of uncrosslinked and crosslinked PPF/mPOSS samples have been performed. It has been found that mPOSS can behave as a crosslinker to enhance the photo-crosslinking efficiency of PPF, regulate mechanical properties, decrease the zero-shear viscosity for both HPPF and LPPF, and accelerate the hydrolytic degradation of the crosslinked PPF/mPOSS networks in NaOH solution, which could be of great interests for applications in fabrication of bone scaffolds and bone repair. No role of mPOSS has been revealed in influencing surface chemistry of crosslinked PPF/mPOSS as its surface hydrophilicity and protein adsorption did not vary significantly from crosslinked PPF. Further, we have evaluated the *in vitro* cytocompatibility of photo-crosslinked PPF/mPOSS samples for bone regeneration using mouse MC3T3 cells and found that photo-crosslinked PPF/mPOSS disks did not demonstrate cytotoxicity in 4 days. Similar to crosslinked PPF, crosslinked PPF/mPOSS disks could support MC3T3 cell attachment, spreading, proliferation, and differentiation and showed no significant difference among the samples with different compositions. However, enhanced cell attachment, proliferation, ALP activity, calcium content, and

upregulation of both osteocalcin and ALP genes have been found on crosslinked HPPF/mPOSS blends compared to their LPPF/mPOSS counterparts, indicating that PPF molecular weight can influence MC3T3 cell behavior, possibly through the different surface stiffnesses in crosslinked PPF.

**Chapter 3. Distinct Cell Responses to Substrates Consisting of
Poly(ϵ -caprolactone) and Poly(propylene fumarate) in the Presence or
Absence of Crosslinks**

3.1 Introduction

Among material properties, surface physicochemical characteristics such as chemical composition, hydrophilicity, morphology, and stiffness have been found to determine cell-biomaterial interactions [6-8]. Many polymeric systems have been investigated in terms of regulating cell behavior through controllable surface properties [159-161]. In particular, much attention has been focused on hydrogels and hydrophilic polymers with tunable surface chemistry and mechanical properties [159-161]. For hydrophobic polymer networks such as silicone rubber, the role of crosslinking density has also been found in regulating mechanical properties and cell behavior [162-164]. Recently we reported the strategy of combining crystallinity and crosslinking density together to achieve non-monotonic or parabolic dependence of substrate properties and cell behavior [39]. In order to better understand the role of crosslinking density, here we present a comparative study using a polymer blend system consisting of two biodegradable polymers, PPF and PCL, in the presence or absence of crosslinks. In both uncrosslinked and crosslinked blends studied here, substantial crystallinities still remained and they were comparable at the same composition. This research itself can also be considered as a comparative study for the investigations on polymer networks covalently linking crystalline domains that are more significantly suppressed by the crosslinks [36,39,55-57,83].

In this comparative study, we have investigated both uncrosslinked PPF/PCL blends

and the photo-crosslinked semi-interpenetrating networks (semi-IPNs) from these blends. In the semi-IPNs, a chemically crosslinked PPF network is interterwined with a physical network associated by the PCL crystalline domains without covalent connections to form pseudo-IPNs. This method was also applied by photo-crosslinking poly(ethylene glycol) dimethacrylate (PEGDA) with poly(D,L-lactic acid) (PDLLA) as a facile and efficient approach to achieve biomaterials with a board spectrum of properties [165]. Unlike these PEGDA-based hydrogels [165], the semi-IPNs in the present study were hydrophobic and not water-swellaable. We have also studied how these two co-existing networks collectively determine the thermal, mechanical, and rheological properties as well as surface hydrophilicity and morphology. As a comparison, uncrosslinked PPF/PCL blends have also been evaluated to demonstrate the opposite composition dependence of mechanical properties.

Aiming for the clinical purpose in hard tissue engineering application exemplified by bone regeneration [166,167], we deliberately selected mouse MC3T3 cells for studying cell behavior. We have evaluated the cytotoxicity of the crosslinked and uncrosslinked samples and characterized cell adhesion, spreading, and proliferation on the 2D substrates made from these samples. Therefore, this study not only supplies a series of biodegradable polymer blends with efficiently controllable properties for tissue engineering applications but also offers a model system for examining the roles of crosslinks and crystallinity in determining both material characteristics and cell behavior.

The results are presented in the following order: bulk properties, surface properties, and cell behavior. Further discussion on cell-material interactions and surface segregation is given by comparing uncrosslinked and crosslinked PPF/PCL blends.

3.2 Experimental Section

3.2.1 Materials

The molecular weights of PPF and PCL used in this study were determined by using an integrated Gel permeation chromatography (GPC) system (PL-GPC 20, Polymer Laboratories, Inc.) at room temperature. PPF synthesized using the previous method had a number-average molecular weight (M_n) of 2000 g/mol and a weight-average molecular weight (M_w) of 2800 g/mol [24,37-39]. PCL ($M_n = 98,000$ g/mol, $M_w = 144,000$ g/mol from GPC) was purchased from Aldrich Co. (Milwaukee, WI) with a nominal molecular weight of 80,000 g/mol. Photo-initiator, phenyl bis(2,4,6-trimethyl benzoyl) phosphine oxide (BAPO, IRGACURE819) was a gift from Ciba Specialty Chemicals (Tarrytown, NY).

3.2.2 Preparation of Uncrosslinked and Crosslinked Samples

For the ease of processing, PPF, PCL, and their blends with ϕ_{PPF} of 75%, 50%, and 25% were prepared by fully dissolving PPF and PCL in methylene chloride (CH_2Cl_2) with different total polymer concentrations, 1.5 g in 0.6, 7, 2, 4, and 6 mL, respectively. For uncrosslinked samples except PPF, films were made by gently pouring polymer

solution into a glass petri-dish and dried slowly in a fume hood. For photo-crosslinked samples, 75 μL BAPO/ CH_2Cl_2 solution (300 mg BAPO in 1.5 mL CH_2Cl_2) was mixed with the polymer solution (1.5 g polymer in different amounts of CH_2Cl_2). Then the polymer/BAPO/ CH_2Cl_2 mixture was poured into a glass petri-dish and placed under the ultraviolet (UV) light ($\lambda = 315\text{-}380$ nm) generated from a Spectroline high-intensity long-wave UV lamp (SB-100P, Intensity: 4800 uw/cm^2) for 30 min. Crosslinked PPF disks (9×1 mm, diameter $\times$ thickness) were prepared separately using a mold formed by two glass plates and a silicone rubber spacer. Other polymer disks ($9 \times 0.16\text{-}0.31$ mm, diameter $\times$ thickness) for characterizations and cell studies were cut from the crosslinked films using a cork-borer after being completely dried in a vacuum oven.

3.2.3 Structural Characterization

Fourier-transform infrared spectroscopic (FTIR) spectra were obtained on a Perkin Elmer Spectrum Spotlight 300 spectrometer with Diamond Attenuated Total Reflectance. The resolution of the instrument was specified as 4 cm^{-1} at the wavenumber of 1000 cm^{-1} . For determining gel fractions, crosslinked polymer films placed in four filter paper bags were extracted using CH_2Cl_2 for 24 h in a Soxhlet extraction apparatus at $60\text{ }^\circ\text{C}$. Finally they were dried in a vacuum oven over night and weighed. Based on the measured weights of the original (W_0) and dry samples (W_d), the gel fractions of crosslinked PPF and PPF/PCL samples were calculated using the equation of $W_d/W_0 \times 100\%$.

3.2.4 Thermal Properties

Differential Scanning Calorimetric (DSC) measurements were performed on a Perkin Elmer Diamond differential scanning calorimeter in a nitrogen atmosphere. To keep the same thermal history, each sample was first heated from room temperature to 100 °C and cooled to –90 °C at a cooling rate of 5 °C/min. Then a subsequent heating run was performed from –90 °C to 100 °C at a heating rate of 10 °C/min. DSC scans for the last cooling and heating runs were recorded for analysis. Thermogravimetric analysis (TGA) was conducted using a Mettler-Toledo Instruments thermal analyzer in flowing nitrogen at a heating rate of 20 °C/min.

3.2.5 Rheological and Mechanical Properties

Linear viscoelastic properties of PCL and its blends with PPF were measured by a strain-controlled rheometer (RDS-2, Rheometric Scientific) at frequencies from 0.5 to 100 rad/s and at various temperatures from 60 to 140 °C. Oscillatory shear measurements were conducted using a parallel plate flow cell (25 mm, diameter; ~0.5 mm, gap) to determine storage and loss moduli G' and G'' , and zero-shear viscosity η_0 . Mechanical measurements were performed at both room temperature and 37 °C. Tensile properties of uncrosslinked and crosslinked PPF/PCL films were evaluated using a dynamic mechanical thermal analyzer (DMTA-V, Rheometric Scientific). Briefly, polymer strips (~20 × ~2 × 0.16-0.31 mm, length × width × thickness) were pulled at a strain rate of 0.001 s⁻¹. Force-displacement curves were converted to stress–strain plots and elastic moduli were calculated from the initial slopes in the plots. At least five specimens for

each sample were measured and averaged.

3.2.6 Water Contact Angle Measurement and Protein Adsorption

Surface morphology of crosslinked PPF/PCL disks was characterized using a multimode atomic force microscope (AFM) with a Nanoscope III control system (Veeco Instruments, Santa Barbara, CA). Images were acquired using tapping mode at room temperature with a scan size of $20 \times 20 \mu\text{m}$ at a scan rate of 0.5 Hz. Surface topography was recorded simultaneously with a standard silicon tapping tip on a beam cantilever and the root mean square (rms) roughness was calculated from height profiles. Water contact angle measurements were performed using a Ramé-Hart NRC C. A. goniometer (model 100-00-230, Mountain Lakes, NJ) after a static time of 30 s. Approximately one drop of 10 μL de-ionized water ($\text{pH} = 7.0$) was injected onto the film surface and a tangent method was used to determine the contact angle in degrees. For each composition, three disks were used and six values were taken for calculating the average value and standard deviation. For protein adsorption, thin disks (9 mm, diameter) were immersed in α -Minimum Essential Medium (Gibco, Grand Island, NY) for 4 h at 37 °C which was supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco). Then these samples were transferred into 48-well plates (one disk per well) and 500 μL of phosphate buffered saline (PBS) was used to wash the disks three times. Ten minutes of gentle agitation was applied and PBS was discarded after each wash. Then 350 μL of 1% sodium dodecyl sulfate (SDS) solution was put into these wells for 1 h at

room temperature. This step was repeated twice and the SDS solution was collected in a plastic vial. The protein concentrations in the collected SDS solutions were determined on a microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA) using a MicroBCA protein assay kit (Pierce, Rockford, IL). Absolute quantification was achieved using a standard curve constructed on Albumin solutions in SDS with eight known concentrations.

3.2.7 Cell Culture and *In Vitro* Cytocompatibility

Mouse MC3T3-E1 pre-osteoblast cells (CRL-2593, ATCC, Manassas, VA) were used to evaluate the cytocompatibility of the uncrosslinked and crosslinked samples. The cells were plated on regular tissue culture flasks with cell culture media in the 37 °C incubator for 24 h prior to cytotoxicity assays. Polymer disks were sterilized in excess 70% ethanol solution overnight and dried in a vacuum oven for 24 h then washed by PBS at least three times prior to use. Cytotoxicity evaluation was carried out by seeding MC3T3 cells at a density of 2×10^4 cells/cm² with 600 µL of primary medium in a 24-well plate. The sterilized polymer samples were placed into the trans-wells and immersed in culture medium for exposure to the cells for 1, 2, and 4 days. Empty wells and wells seeded at the same density but no exposure to the polymer samples were negative and positive controls. UV absorbance at 490 nm was determined on the incubated MTS assay solution (CellTiter 96 Aqueous One Solution, Promega, Madison, WI) on a micro-plate reader. Cell viability was evaluated by normalizing the absorption

values to the positive control value.

3.2.8 *In Vitro* Cell Attachment, Spreading, and Proliferation

MC3T3 cell attachment and proliferation were examined on the films of both uncrosslinked and crosslinked PPF/PCL blends. Sterile thin films were attached on the bottom of 48-well plates using autoclave-sterilized inert silicon-based vacuum grease (Dow Corning, Midland, MI) to avoid floating in the culture media. MC3T3 cells were seeded onto the polymer thin films at densities of 1.2×10^4 cells/cm² with 400 μ L of culture medium in each well. Tissue culture polystyrene (TCPS) wells seeded with cells at the same density. Cells were cultured in a humidified atmosphere of 5% CO₂ at 37 °C for 4 h, 1, 2, and 4 days. The attached MC3T3 cells were fixed using 16% paraformaldehyde (PFA) solution for 10 min, washed twice using PBS and permeabilized with 0.2% Triton X-100. Then the cells were stained with rhodamine-phalloidin for 1 h at 37 °C for photographing. The morphology of attached cells was observed and recorded by using an Axiovert 25 Zeiss light microscope (Carl Zeiss, Germany). The software ImageJ (National Institutes of Health, Bethesda, MD) was used to measure average cell area over 20 non-overlapping cells at 4 h and day 1 post-seeding.

3.2.9 Statistical Analysis

Student's *t*-test was performed to assess the statistical significance ($p < 0.05$) of the difference between results.

3.3 Results and Discussion

3.3.1 Structural Characterizations

The chemical structures of PPF, PCL, and their blends were supported by the FTIR spectra in Figure 3.1. The spectra of the blends contained the characteristics of both components. The absorption peaks attributed to methylene groups ($-\text{CH}_2-$) in PCL backbone were prominent at 2950 cm^{-1} while the absorption peak for carbon-carbon double bonds ($-\text{CH}=\text{CH}-$) in PPF-containing samples was shown at 1650 cm^{-1} . The intensities of these two characteristic absorption peaks in the blends showed the dependence on ϕ_{PPF} . After crosslinking, FTIR spectra (Figure 3.1b) did not change much as the carbon-carbon double bonds were only consumed partially in the reaction, in agreement with photo-crosslinked PPF/PCLF and PPF/PCLDA blends reported earlier [36,39].

3.3.2 Rheological Properties

Figure 3.2 demonstrates the rheological properties of PCL and three PPF/PCL blends with ϕ_{PPF} of 25%, 50%, and 75% at temperatures higher than the T_m values of these samples (Table 3.1). Because of the oligomeric characteristics of PPF used in this study, no break-down of time-temperature superposition was found when the curves at five temperatures were shifted to the reference temperature of $140\text{ }^\circ\text{C}$ for constructing master curves, although PPF and PCL were two different polymer species. PCL used in this study was entangled as indicated by the crossover point of G' and G'' in the frequency (ω)

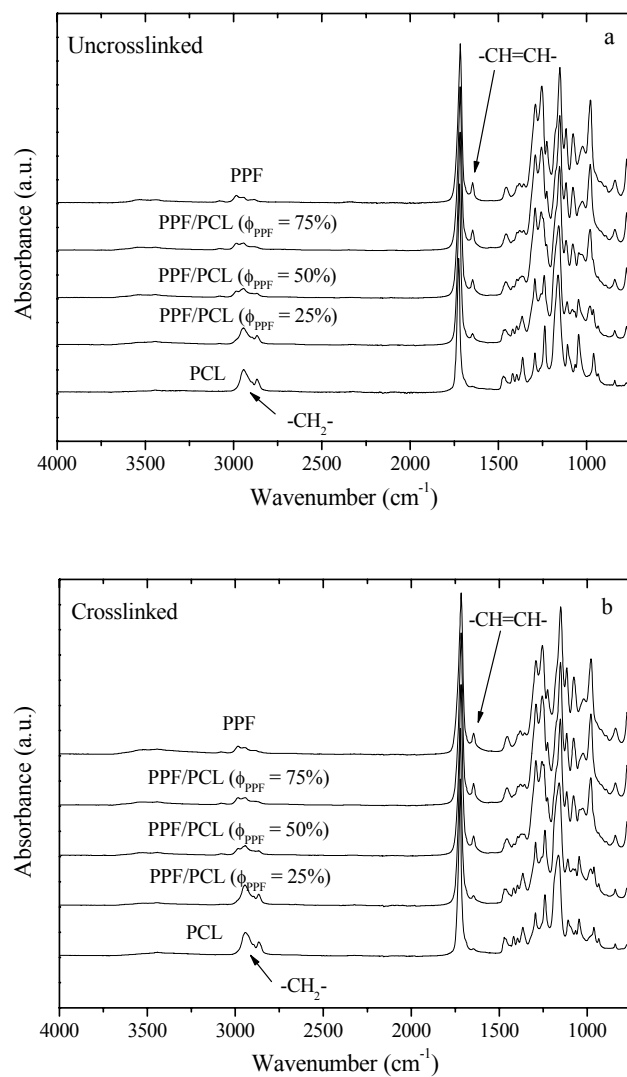


Figure 3.1. FTIR spectra of the uncrosslinked (a) and crosslinked (b) samples.

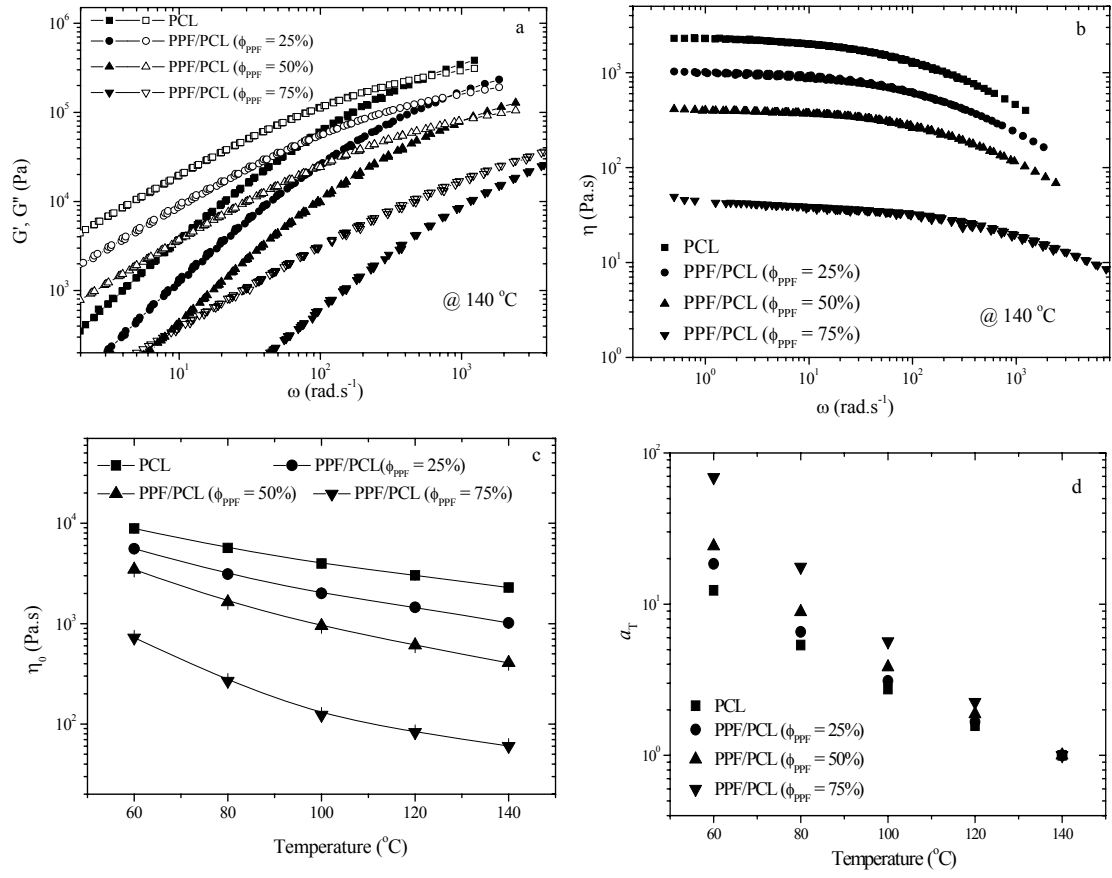


Figure 3.2. Master curves of (a) G' and G'' , (b) viscosity η of PCL and PPF/PCL blends at 140 °C. The temperature dependence of zero-shear viscosity η_0 (c) and horizontal shift factor a_T (d).

range in Figure 3.2a. Measurements at temperatures lower than 60 °C were prohibited by inevitable crystallization of PCL. In the terminal or flow regime at low frequencies, all the polymer samples showed typical scaling relations with ω in G' and G'' as $G' \sim \omega^2$ and $G'' \sim \omega^1$ [168].

The crossover point of G' and G'' marked the transition from fluid behavior to rubbery behavior that is characteristic of G' being always greater than G'' although the rubbery regime was not fully obtained [168]. When ϕ_{PPF} increased, the crossover frequency (ω_c) of G' and G'' shifted from 590 rad/s for PCL to higher values of 800 and 1045 rad/s for PPF/PCL blends with ϕ_{PPF} of 25% and 50%, respectively. The change in ω_c indicated that the longest relaxation time ($\tau \sim 1/\omega_c$) for PCL decreased with adding oligomeric PPF [168]. Meanwhile, the crossover modulus G_c decreased from 2.55×10^5 to 1.51×10^5 and 0.83×10^5 Pa because of the effect of dilution. The G_c values for the blends were lower than the values of 1.35×10^5 and 0.55×10^5 Pa predicted by the empirical equation of $G_c(\phi) \sim \phi^{2.2}$ because PCL used here was polydisperse ($M_w/M_n = 1.47$) and the blends were only barely entangled [168-171]. For PPF/PCL ($\phi_{\text{PPF}} = 75\%$), its master curve did not have a crossover point to indicate the entanglement in the frequency range studied. The corresponding viscosity curves for these uncrosslinked samples are shown in Figure 3.2b. Zero-shear viscosity (η_0) was obtained from the broad Newtonian region where viscosity was independent of frequency. The zero shear viscosity at 140 °C decreased significantly from 2320 Pa.s for PCL to 1020, 408, and 42

Pa.s when 25%, 50%, and 75% PPF was added because the shorter PPF chains can be considered as a diluent for the longer PCL chains. The composition dependence of η_0 followed the power law with a scaling exponent of 2.87, which was slightly lower than the value of 3.3 for polymer concentrated solutions in small molecular solvents [168-171].

Figure 3.2c shows that the temperature dependence for η_0 of PCL and three PPF/PCL blends at temperatures of 60, 80, 100, 120, and 140 °C. The temperature dependence of viscosity became stronger when ϕ_{PPF} increased, consistent with the earlier findings on the copolymers and blends containing PPF and PCL components [36,37,39]. The same trend can be also observed in the temperature dependence of the horizontal shift factor a_T (Figure 3.2d) rendered from constructing the master curves of G' and G'' in Figure 3.2a. The Williams-Landel-Ferry (WLF) equation was used to model the temperature dependence of viscosities, especially at $T_g + 20\text{ °C} < T < T_g + 100\text{ °C}$ [168].

$$\log \frac{\eta(T)}{b_T \eta(T_0)} = -\frac{C_1(T-T_0)}{C_2 + (T-T_0)} \quad (1)$$

In eq. 1, C_1 (dimensionless) and C_2 (in K) are two constants, b_T is the vertical shift factor, and T_0 is the reference temperature [168]. b_T can be safely ignored here because it depends very weakly on temperature and is close to unity [168]. The temperature dependence of η_0 in Figure 3.2c can be fitted perfectly using the WLF equations with a squared correlation coefficient R^2 of 0.9992 when T_0 was set as 60 °C. The constant C_1 in

eq. 1 increased continuously from 1.72 for PCL to 1.86, 2.27 and 2.35 for PPF/PCL at ϕ_{PPF} of 25%, 50%, and 75%, respectively. Correspondingly, C_2 decreased from 157.5 to 126.9, 118.4, and 88.5 °C.

3.3.3 Gel Fraction

After photo-crosslinking, PPF formed a chemical network with a fairly high density of crosslinking while PCL did not covalently participate in the PPF network. The gel fraction of the crosslinked samples was used to evaluate the efficiency of PPF photo-crosslinking in the blends. As shown in Figure 3.3, the experimental gel fractions measured using extraction in CH_2Cl_2 were lower than the “theoretical” values indicated by the diagonal line from zero to one. The prediction was made based on the assumption that the PPF moiety in the blends could crosslink into a perfect network with a gel fraction of 100%. The ratio of the experimental value to the theoretical value, i.e. ϕ_{PPF} , increased gradually from 0.775 to 0.841, 0.914, and 0.952 when ϕ_{PPF} increased from 0.25 to 0.50, 0.75, and 1, respectively. This result was expected because PCL started to crystallize during the photo-crosslinking and the opaqueness from crystallization might block UV light from penetrating into the blends to initiate crosslinking efficiently. Nevertheless, the gel fractions were still sufficient for forming PPF networks with integrity in the semi-IPNs.

3.3.4 Thermal Properties

The DSC curves of both uncrosslinked and crosslinked samples recorded in the

Table 3.1. Thermal properties of uncrosslinked and crosslinked (x) PPF/PCL blends

ϕ_{PPF}	$T_m(T_{cc})$	$\Delta H_m(\Delta H_{cc})$	T_c	ΔH_c	T_d	Residue	χ_{ac}	χ_c
	(°C)	(J/g)	(°C)	(J/g)	(°C)	(%)	(%)	(%)
100%	---	---	---	---	324	11.4	0	0
75%	51.2 (10.0)	18.6 (12.8)	---	---	335	9.5	13.8	55.1
50%	52.8	37.1	12.7	36.5	374	6.3	27.5	54.9
25%	53.7	45.9	21.5	47.5	388	3.9	34.0	45.3
0	56.0	56.1	24.9	55.1	400	1.0	41.6	41.6
100% (x)	---	---	---	---	342	10.1	0	0
75% (x)	54.6	14.5	24.0	13.8	334	8.2	10.7	42.9
50% (x)	54.8	27.8	25.2	28.9	371	5.6	20.6	41.2
25% (x)	54.6	41.8	28.7	43.2	384	2.8	30.1	41.3

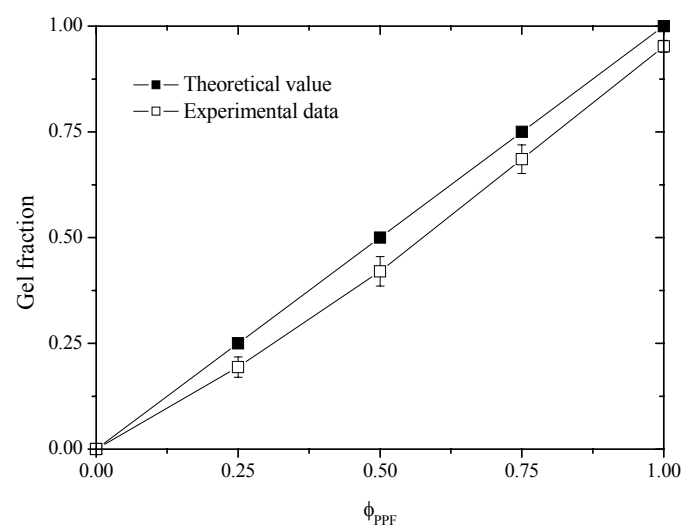


Figure 3.3. Gel fractions of crosslinked PPF and PPF/PCL blends (solid squares: theoretical values; open symbols: experimental values).

heating and cooling runs are shown in Figure 3.4. There was only a glass transition at T_g of -0.2 °C for PPF while PCL was a semi-crystalline polymer with a very low T_g of ~-60 °C but a melting peak at ~55 °C. Glass transition was not discernible for the blends from the DSC curves due to the high crystallinity in PCL. T_m and the heat of fusion (ΔH_m) were determined from the melting peak in the heating run while the crystallization temperature T_c and the corresponding heat of fusion (ΔH_c) were from the crystallization peak in the cooling run. An exothermal peak with the heat of fusion (ΔH_{cc}) of 12.8 J/g was observed at a cold crystallization temperature (T_{cc}) of 10.0 °C in the heating run for crosslinked PPF/PCL ($\phi_{PPF} = 75\%$). These thermal properties are listed in Table 3.1.

Without crosslinking, T_m decreased progressively by adding amorphous PPF from 56.0 °C for PCL to 51.2 °C for PPF/PCL with ϕ_{PPF} of 75%. ΔH_m decreased with ϕ_{PPF} in both uncrosslinked and crosslinked PPF/PCL blends. Because PPF networks suppressed the crystalline domains of PCL, the ΔH_m values for the crosslinked PPF/PCL blends were significantly lower than those uncrosslinked ones at the same compositions. The crosslinked PPF/PCL blends were very distinct from the polymer networks where crystallizable PCL blocks were covalently connected from both ends by the crosslinks and consequently the crystallinity can be reduced more significantly or even completely [36,39,55-57,83]. Another parameter, T_m no longer varied with the composition after crosslinking because T_m was related to the lamellar thickness in PCL and the relatively stationary PPF network did not interact with the PCL chains as mobile individual chains.

As shown in Figure 3.4b and Table 3.1, T_c and ΔH_c both decreased with increasing ϕ_{PPF} . In addition, T_c decreased more for the uncrosslinked blends and the crystallization peak disappeared at ϕ_{PPF} of 75%. T_c was lower than T_m because of supercooling that was required for crystallization and slower cooling. The crystallinity χ_c for PCL moiety in the blends was calculated from their ΔH_m values using the equation of $\chi_c = \frac{\Delta H_m}{\phi_{PCL} \cdot \Delta H_m^c} \times 100\%$ with the known ΔH_m^c value of 135 J/g for completely crystalline PCL [172], and the PCL composition (ϕ_{PCL}) in the blends, i.e., $1 - \phi_{PPF}$. In the semi-IPNs, χ_c did not change while it increased with adding more PPF. The apparent crystallinity χ_{ac} for the blend was also calculated using the equation of $\chi_{ac} = \frac{\Delta H_m}{\Delta H_m^c} \times 100\%$ and listed in Table 3.1, showing the same trend as ΔH_m .

In order to evaluate the thermal stability of the samples in processing and verify the blend composition, TGA thermograms of both uncrosslinked and crosslinked PPF/PCL blends are shown in Figures 3.5a and 3.5b. There was one single degradation step and the onset thermal degradation temperature T_d decreased progressively with ϕ_{PPF} . However, two separate peaks with intensities proportional to the composition of the ascribed component were observed when derivative of weight change was plotted against temperature in Figures 3.5c and 3.5d to indicate the existence of two distinct components in the blends. Meanwhile, the residue fraction at 550 °C was 1.0% for pure PCL and it increased proportionally with ϕ_{PPF} up to as high as 10.3% for pure PPF.

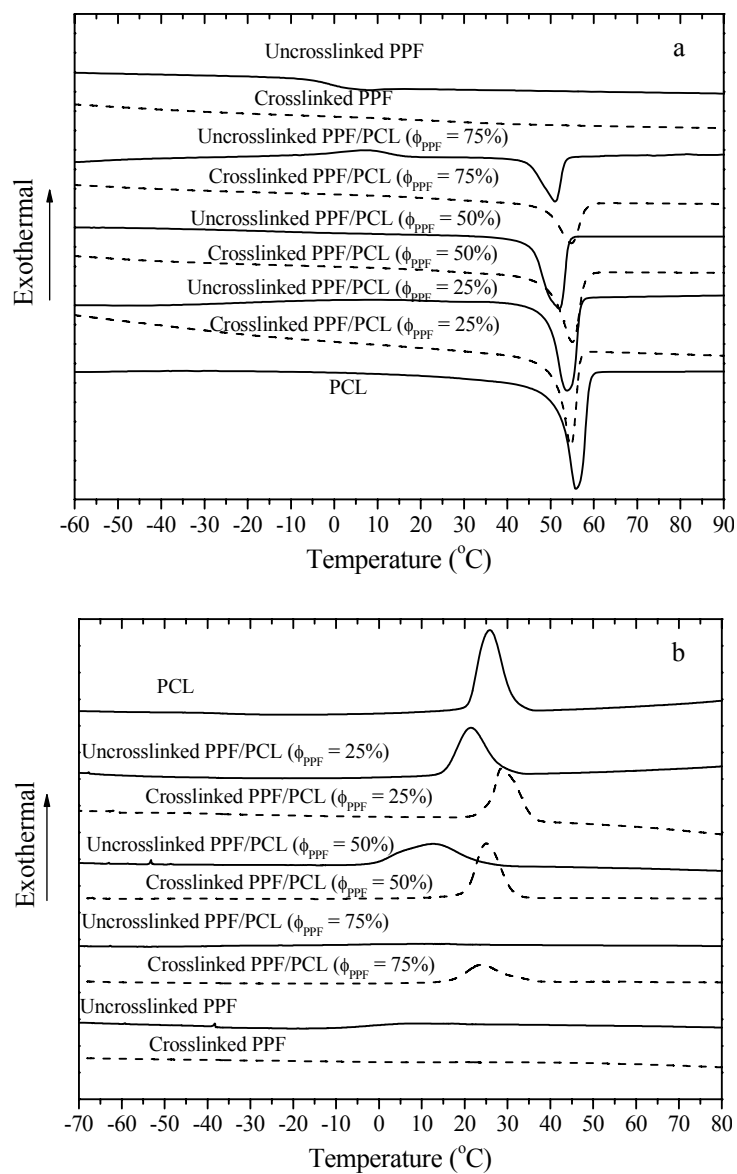


Figure 3.4. DSC curves of the uncrosslinked (solid curves) and crosslinked (dotted curves) samples recorded in the scans of (a) heating and (b) cooling.

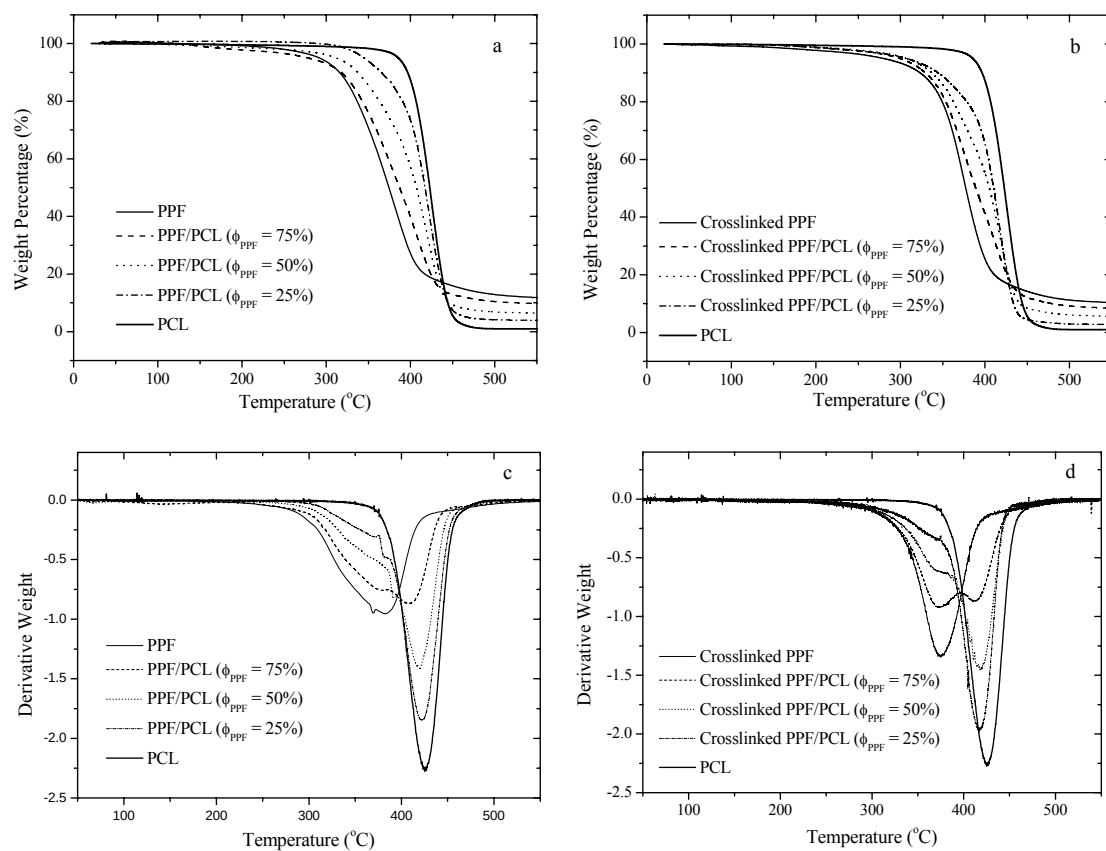


Figure 3.5. TGA thermograms (a, b) and derivative thermogravimetric (DTG) curves (c, d) of the uncrosslinked (a, c) and crosslinked (b, d) samples.

3.3.5 Mechanical Properties

Both crystallinity and crosslinking density determine the mechanical properties of a polymer network while crosslinks suppress crystallinity. By crosslinking PPF with this high-molecular-weight crystalline PCL, material properties can be tuned using the double networks of the PCL crystalline domains interpenetrating in the photo-crosslinked PPF networks. As shown in Figure 3.6, the tensile modulus at room temperature increased with ϕ_{PPF} dramatically from 526 ± 12 MPa for crosslinked PPF/PCL ($\phi_{\text{PPF}} = 25\%$) to 665 ± 52 MPa, 858 ± 62 MPa, and 1.48 ± 0.05 GPa for crosslinked PPF/PCL blends with ϕ_{PPF} of 50% and 75%, and crosslinked PPF, respectively. On the contrary, the modulus for the uncrosslinked samples decreased progressively with ϕ_{PPF} from 388 ± 24 MPa for PCL to 166 ± 6 , 106 ± 15 , and 38 ± 2.6 MPa for PPF/PCL blends with ϕ_{PPF} of 25%, 50%, and 75%, respectively. It was expected because PPF used here was amorphous with a T_g lower than the room temperature and therefore lacked mechanical strength. Unlike those semi-crystalline networks based on PCLF, PCLDA, PPF-co-PCL, PPF/PCLF, or PPF/PCLDA blends [36,39,55-57,83], crosslinking density was the dominant role in determining the mechanical properties of crosslinked PPF/PCL blends because the crosslinked samples have slightly lower crystallinities compared to their uncrosslinked counterparts (Table 3.1). At 37 °C, all the tensile moduli were 40-55% lower than the values at room temperature except the crosslinked PPF, which had no evident thermal transition in this temperature change.

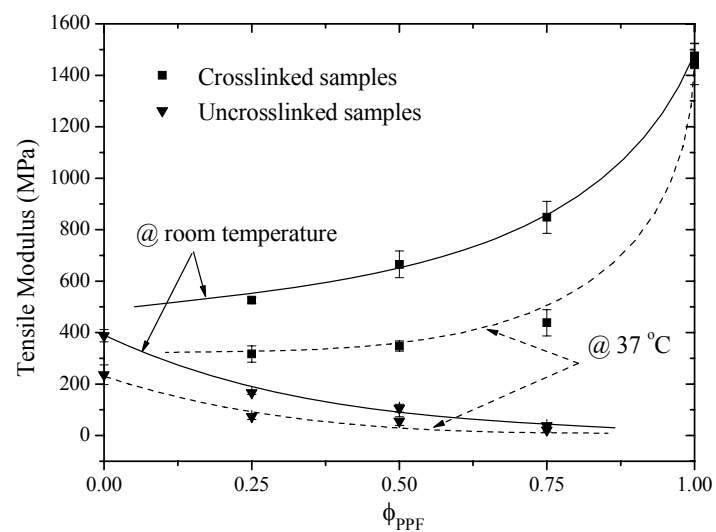


Figure 3.6. Tensile modulus of the uncrosslinked and crosslinked samples at room temperature (solid curves) and at 37 °C (dotted curves).

The stress at break at 37 °C decreased significantly from 27.2 ± 1.8 MPa for crosslinked PPF to 10.5 ± 1.0 MPa for crosslinked PPF/PCL ($\phi_{\text{PPF}} = 75\%$) while the strain at break increased from $2.7 \pm 0.3\%$ to $5.4 \pm 0.5\%$. For uncrosslinked PPF/PCL ($\phi_{\text{PPF}} = 75\%$), the stress at break was much lower (0.7 ± 0.3 MPa) and the strain at break was similar ($6.7 \pm 0.5\%$) at 37 °C. At $\phi_{\text{PCL}} \geq 50\%$, both uncrosslinked and crosslinked PPF/PCL blends were tougher and showed necking phenomenon in tensile elongation. No break was found for these samples when elongated in the displacement range of the instrument, which resulted in a maximum strain of 10%. From the above results, it is evident that mechanical properties can be modulated by controlling the blend composition. The role of crosslinks in mechanical properties was critical but different from that in the networks where crystallizable segments covalently participated in the chemical crosslinking [36,39,55-57,83].

3.3.6 Surface Characteristics

Surface topography and surface chemistries such as hydrophilicity and the capability of adsorbing proteins from cell culture media are important properties to influence cell-material interactions. AFM images were taken on crosslinked samples and PCL. Because the crystalline structure of PCL induced topological heterogeneity, it can be observed from Figure 3.7 that the surfaces of crosslinked PPF/PCL blends became rougher when ϕ_{PPF} decreased. rms surface roughness was similar for crosslinked PPF (8.3 ± 0.3 nm) and PPF/PCL blends with ϕ_{PPF} of 75% (7.1 ± 0.9 nm) and 50% (8.0 ± 0.5 nm).

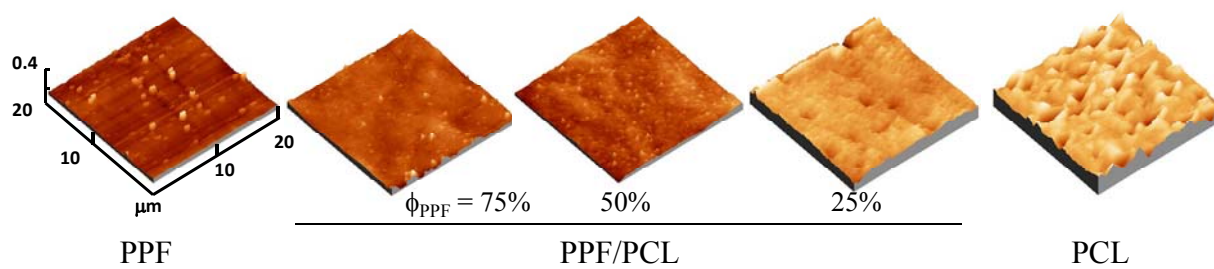


Figure 3.7. AFM images of crosslinked PPF and PPF/PCL blends as well as PCL.

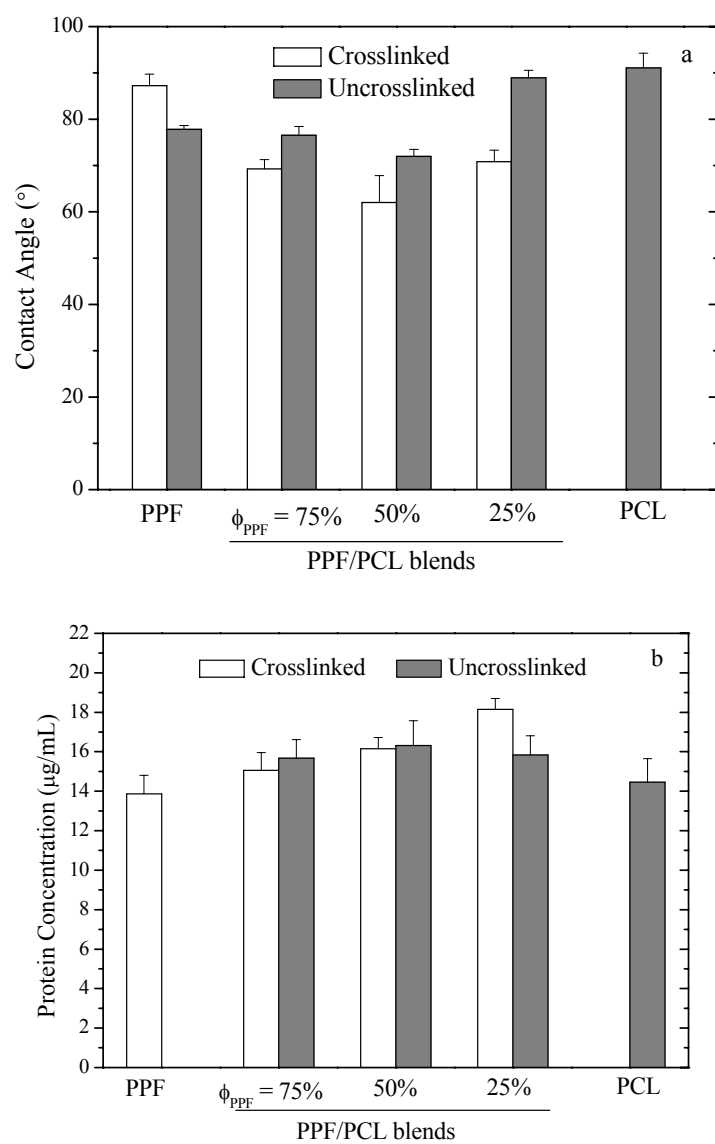


Figure 3.8. Water contact angles (a) and the concentration of proteins adsorbed (b) on the uncrosslinked and crosslinked samples.

It increased significantly to 38.3 ± 5.1 nm for PPF/PCL blends with ϕ_{PPF} of 25% and eventually to 188.3 ± 22.1 nm for PCL. Uncrosslinked PPF and PPF/PCL samples were too tacky to be characterized using AFM.

As reported in literature, material surface hydrophilicity or surface energy determines the wettability of fabricated biomedical devices and the capability of a surface to adsorb proteins from culture medium as well as cell adhesion [6-8,38]. The result in Figure 3.8a showed that the blend films were more hydrophilic than both PPF and PCL. The water contact angle reached minimum values of $62.0 \pm 5.8^\circ$ and $72.0 \pm 1.5^\circ$ for crosslinked and uncrosslinked PPF/PCL disks at ϕ_{PPF} of 50%, respectively. A clear difference should be noted between the uncrosslinked and crosslinked samples. The polymer samples became slightly more hydrophilic after crosslinking possibly because the crosslinked samples had lower crystallinities and smoother surfaces. As shown in Figure 3.8b, the concentration of adsorbed proteins on the disks decreased with ϕ_{PPF} from 18.1 ± 0.6 $\mu\text{g/mL}$ for crosslinked PPF/PCL ($\phi_{\text{PPF}} = 25\%$) to 13.9 ± 0.9 $\mu\text{g/mL}$ for crosslinked PPF. In contrast, the values for the uncrosslinked samples varied only slightly from 14.5 ± 1.2 to 16.3 ± 1.3 $\mu\text{g/mL}$ without showing clear composition dependence. There was a trend that a more hydrophobic surface had a relatively lower amount of adsorbed proteins, which may be attributed to better spreading of cell culture media on more hydrophilic disk surfaces.

3.3.7 *In Vitro* Cell Behavior

Mouse MC3T3–E1 cells were used to evaluate the controllability of the responses to the polymer substrates with varied surface physicochemical characteristics. Cell viability was found to be close to 100% when cultured in the presence of the uncrosslinked and crosslinked samples (Figure 3.9), suggesting these polymer samples did not contain cytotoxic leachable components or degradation products within the time period for cell studies. Then we examined cell attachment and spreading, which is related to gene expression and protein production [6-8,159-161].

In Figure 3.10, cell attachment at 4 h post-seeding is shown after normalization to the average number of attached cells in the positive control wells. It is evident that the responses to the crosslinked samples were distinct from the uncrosslinked counterparts. For the crosslinked samples and PCL, the dependence of MC3T3 cell attachment on composition was not monotonic as it showed a minimum at ϕ_{PPF} of 75%. In clear contrast, cell attachment decreased sharply with increasing ϕ_{PPF} in the uncrosslinked PPF/PCL substrates, particularly when ϕ_{PPF} was larger than 25%. The average area of attached MC3T3 cells at 4 h post-seeding also demonstrated similar composition dependence, as shown in Figure 3.11a. The difference among all those crosslinked PPF/PCL samples became more prominent and cells spread better after one day. For uncrosslinked PPF/PCL ($\phi_{\text{PPF}} = 25\%$) films, MC3T3 cell area was much smaller ($1048 \pm 173 \mu\text{m}^2$ at 4 h and $1577 \pm 144 \mu\text{m}^2$ at day 1) compared with the crosslinked ones.

The fluorescent images of MC3T3 cells on both crosslinked and uncrosslinked

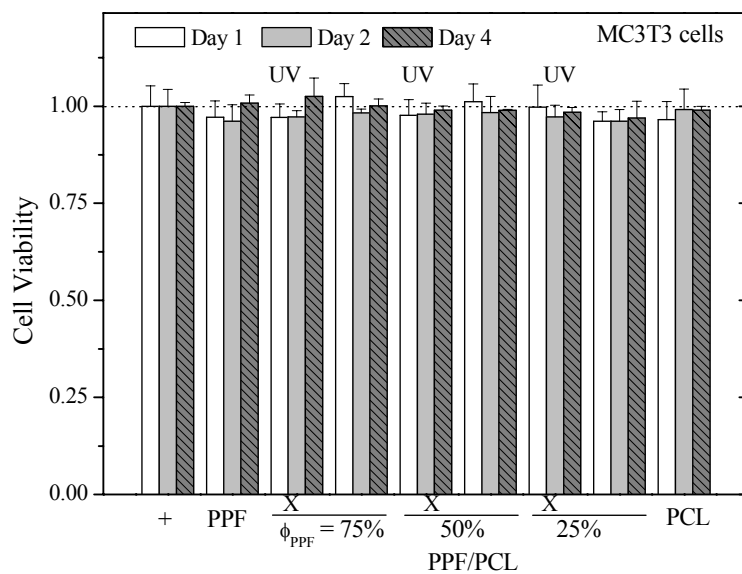


Figure 3.9. MC3T3 cell viability when cultured in the presence of the crosslinked (x) and uncrosslinked samples, indicated by normalizing MTS absorption to the average value of cell-seeded culture polystyrene (TCPS) wells without being exposed to the samples as positive (+) control at 1, 2, and 4 days.

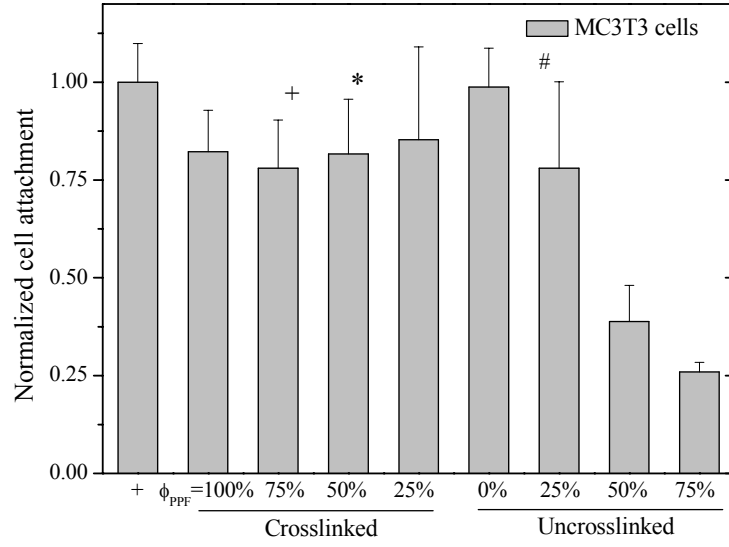


Figure 3.10. Normalized MC3T3 cell attachment 4 h post-seeding on the disks of the uncrosslinked and crosslinked samples with different ϕ_{PPF} , compared with cell-seeded tissue-culture polystyrene (TCPS) as positive control. *, $p < 0.05$ relative to uncrosslinked PPF/PCL ($\phi_{PPF} = 50\%$); +, $p < 0.05$ relative to uncrosslinked PPF/PCL ($\phi_{PPF} = 75\%$); #, $p < 0.05$ relative to uncrosslinked PPF/PCL ($\phi_{PPF} = 75\%$ and 50%).

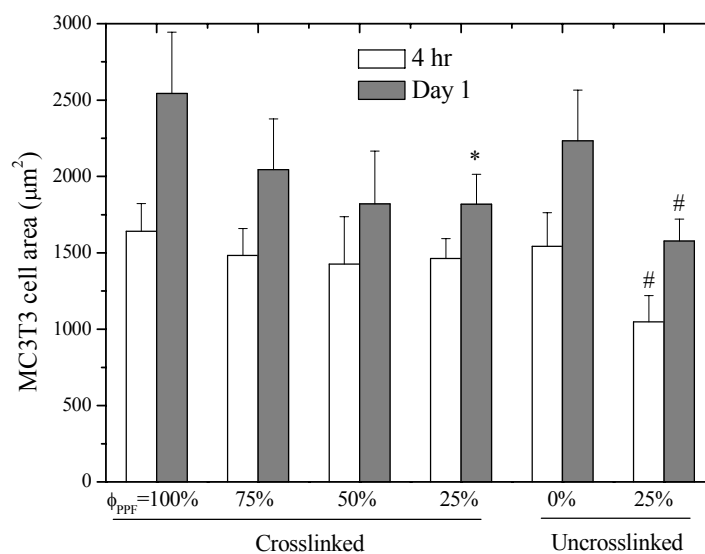


Figure 3.11. Cell area after 4 h and 1 day of crosslinked PPF and PPF/PCL ($\phi_{PPF} = 75\%$, 50%, and 25%), and uncrosslinked PCL and PPF/PCL ($\phi_{PPF} = 25\%$). *, $p < 0.05$ relative to crosslinked PPF and PCL; #, $p < 0.05$ relative to crosslinked PPF and PPF/PCL ($\phi_{PPF} = 75\%$, 50%, and 25%), and PCL.

PPF/PCL samples at 4 h, days 1, 2, and 4 post-seeding and the corresponding cell numbers calculated from MTS absorptions are demonstrated in Figure 3.12. Cell densities found in the images (Figure 3.12a) were consistent with the cell numbers (Figure 3.12b). Similar to cell attachment and spreading, cell proliferation also demonstrated dependence on the substrate composition and the trends in crosslinked and uncrosslinked samples were very distinct. For the uncrosslinked samples, MC3T3 cell proliferation decreased significantly when PPF was added to PCL. When ϕ_{PPF} was equal to or greater than 50% in the uncrosslinked blends, no MC3T3 cell proliferation was found. Only a few cells with a rounded phenotype appeared in the images. In clear contrast, the crosslinked samples were more supportive for cell proliferation. On both PCL and crosslinked PPF, confluent MC3T3 cells were found at day 4 post-seeding. At day 1, the cell number did not demonstrate a significant difference among all the crosslinked blends with varied compositions. At day 2 and day 4, an evident minimum can be seen at ϕ_{PPF} of 50%. Proliferation index (PI) was calculated by dividing the cell number at day 4 by the initial number of attached cells at 4 h post-seeding. The PI of MC3T3 cells decreased from 4.66 ± 0.63 for crosslinked PPF to 4.43 ± 0.82 , 3.47 ± 0.70 , and 3.27 ± 1.03 for crosslinked PPF/PCL blends with ϕ_{PPF} of 75%, 50%, and 25%, respectively. Correspondingly, the doubling time of MC3T3 cells on the crosslinked samples increased from 1.80 ± 0.11 days to 1.86 ± 0.16 , 2.23 ± 0.25 , and 2.34 ± 0.43 days. For the uncrosslinked samples, the PI of MC3T3 cells decreased from 3.99 ± 0.65 for PCL to 2.08 ± 0.47 after adding 25%

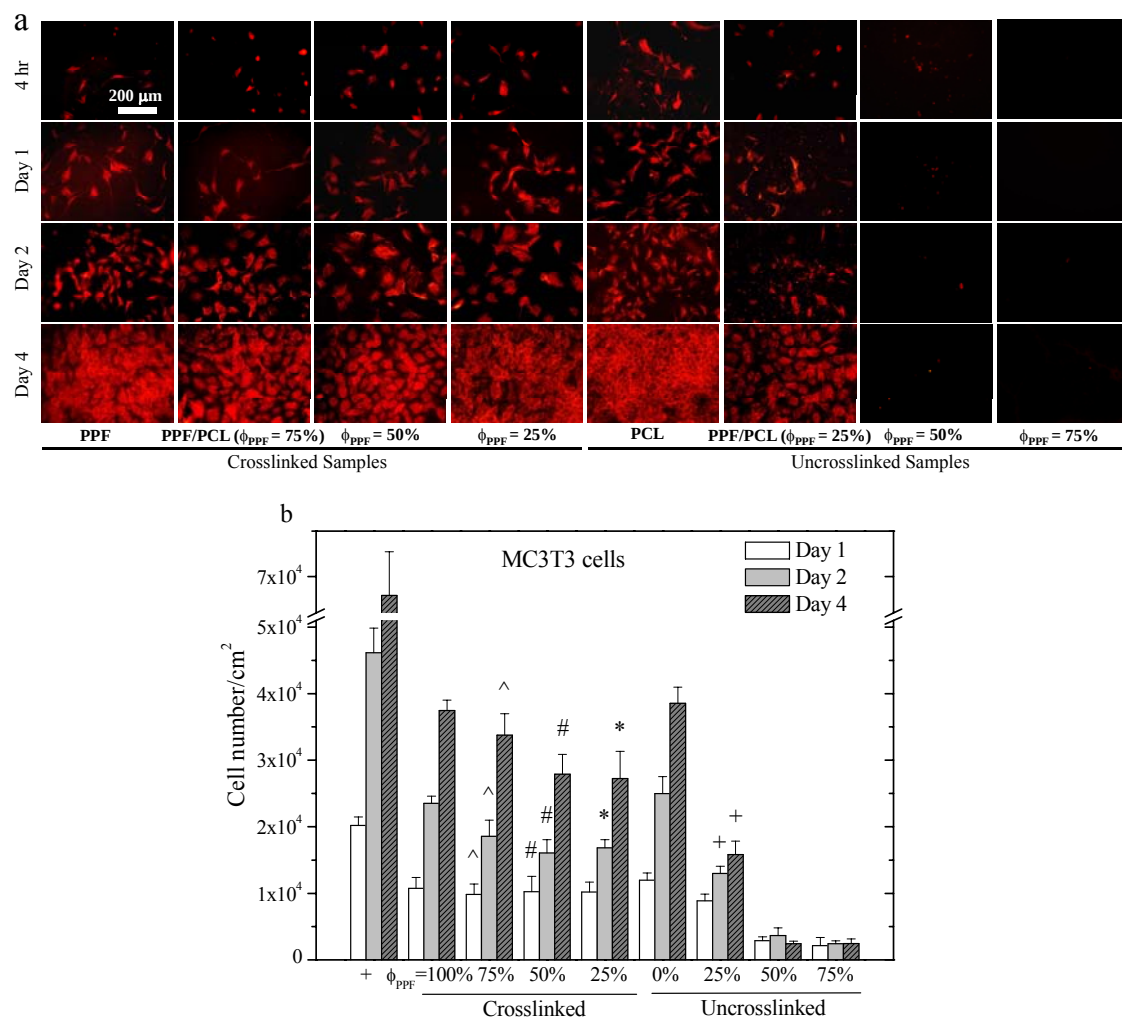


Figure 3.12. Fluorescent images (a) and numbers (b) of cells on crosslinked PPF and PPF/PCL ($\phi_{PPF} = 75\%$, 50%, and 25%), PCL and uncrosslinked PPF/PCL ($\phi_{PPF} = 25\%$, 50%, and 75%) at day 1, 2, 4 post-seeding, compared with TCPS as positive control. *, $p < 0.05$ relative to crosslinked PPF/PCL ($\phi_{PPF} = 100\%$ and 75%), PCL, uncrosslinked PPF/PCL ($\phi_{PPF} = 25\%$), TCPS. +, $p < 0.05$ relative to crosslinked PPF/PCL ($\phi_{PPF} = 25\%$), PCL, uncrosslinked PPF/PCL ($\phi_{PPF} = 75\%$ and 50%). #, $p < 0.05$ relative to uncrosslinked PPF/PCL ($\phi_{PPF} = 50\%$). ^, $p < 0.05$ relative to uncrosslinked PPF/PCL ($\phi_{PPF} = 75\%$).

PPF while the doubling time increased dramatically from 2.00 ± 0.12 to 3.79 ± 1.14 days.

3.4 Further Discussion

The present study supplies a platform for investigating the roles how crystallinity and crosslinking density play in regulating both material properties and cell behavior. In the previous studies on crosslinked PPF/PCLF and PPF/PCLDA blends [36,39], amorphous products can be made when ϕ_{PPF} is sufficiently high or when the PCL molecular weight in PCLF or PCLDA is sufficiently low. Unlike these systems [36,39], crystalline structures always existed in the PPF/PCL blends before and after crosslinking. The role of crosslinks in determining the mechanical properties was emphasized as the crosslinked blends with lower crystallinities were much stiffer than their uncrosslinked counterparts. In the presence or absence of crosslinks, the dependence of tensile modulus on ϕ_{PPF} was contrary because uncrosslinked PPF lacked mechanical strength and was a plasticizer to PCL. Furthermore, crosslinks significantly reduced the mobility of PPF and consequently the semi-IPNs formed by PPF network and PCL crystalline domains were stable at 37 °C.

Crosslinked PPF in this study was more hydrophobic than its uncrosslinked form, as indicated by a difference of 7° in the water contact angle. Crosslinked PPF/PCL blends had lower water contact angles of $\sim 70^\circ$ without a significant difference among compositions. For the uncrosslinked PPF/PCL samples, hydrophilicity was increased by

adding PPF to PCL and again no significant difference could be found when ϕ_{PPF} was higher than 50%. Therefore, the slight difference in hydrophilicity cannot be used to interpret the dramatic variance in cell behavior on the blends in the presence or absence of crosslinks and composition dependence. Further, it is generally believed that a substrate with an intermediate hydrophilicity and a water contact angle of $\sim 50^\circ$ can support cell attachment better than more hydrophobic or more hydrophilic surface [6-8]. On more hydrophilic surfaces, low protein adsorption will occur. On more hydrophobic surfaces, there will be more adsorption of non-adhesive proteins and denaturing of adhesive proteins [6-8]. The weak dependence on the substrate composition found for the proteins adsorbed from the cell culture media could not explain the trend in cell behavior either.

Surface roughness from nanometer to micron length scale can affect cell behavior because the dimensions for individual proteins, integrin clusters, and cells vary in this range [6-8]. Rougher surfaces have more surface area for cells to attach and proliferate. Certain surface patterns such as micro- and nano-grooves can be used to align cells because of the effect of “contact guidance” [103]. Poly(L-lactic acid) (PLLA) and its blends with PDLLA were used to modulate surface roughness and MC3T3 cell proliferation [100,101]. The rate of MC3T3 cell proliferation on smooth surfaces was found to be much greater than that on rougher ones [100,101]. However, the mechanism of this cell response to crystallization-induced surface roughness is still unclear. In this

study, the surfaces of crosslinked PPF/PCL blends were similar in roughness and topography when PPF was dominant. Surface roughness only increased when significant crystallization occurred from the PCL component. Surface erosion during the sterilization in ethanol solution can also cause uneven surfaces. The change in surface roughness for the crosslinked samples and PCL was first independent of and then monotonic with the composition, in disagreement with the trend in the non-monotonic composition dependence of cell attachment and proliferation. Moreover, previously we found that cell attachment and proliferation were not evidently influenced by the surface roughness caused by PCL crystallization in a comparative study using compression-smoothed disks of semi-crystalline photo-crosslinked PCLDA [83]. When uncrosslinked PPF/PCL with a low PPF composition was crystallized at temperatures slightly lower than its T_m , concentric rings with ridges and valleys were observed as the results of banded spherulites [173]. Beyond the scope of the present study, the effect of such spherulite-roughened surfaces and microgroove patterns on cell behavior will be reported soon by our research group.

The role of substrate stiffness in cell-material interactions has attracted great attention since Pelham and Wang reported in 1997 using polyacrylamide (PA)-based hydrogels [6-8,11-13,159-161]. Substrate stiffness influences the cytoskeletal organization and consequently cell phenotype [6-8,11-13,159-161]. Stiffer hydrogels were reported to better support focal adhesion, spreading, proliferation, and

differentiation of MC3T3-E1 cells and human mesenchymal stem cells [174,175]. Wong *et al* reported that SMCs could migrate from soft region to rigid region on gradient-compliant hydrogel substrates [176-178]. Spreading of SMCs was also found to increase asymptotically with substrate stiffness and such response to ECM stiffness was through intracellular stressing mechanisms, e.g., stress fibers and membrane tension [179,180]. A recent study reported that rat aortic SMCs cultured on native polyelectrolyte multi-layers exhibited morphology and motility of synthetic cells while cells cultured on maximally crosslinked substrates displayed contractile phenotype [181]. Another study showed that increase in the nanoscale rigidity of collagen fibrils on collagen thin films can cause vascular SMCs to assume a proliferative phenotype when cultured on these substrates [182]. The present results on MC3T3 cells were in agreement with the previous reports although the substrates had much higher stiffnesses than the hydrogel systems based on PEG or PA and PDMS networks [176-180].

When the substrates were not crosslinked, the crystallinity and T_m of PCL were reduced significantly by adding amorphous PPF with a T_g of -0.2 °C. Because of its low molecular weight, PPF was mobile at 37 °C and could plasticize and significantly soften semi-crystalline PCL with a T_m of ~55 °C. The mechanical properties of the PPF/PCL blends indeed demonstrated monotonic composition dependence. After crosslinking, for the PPF/PCL blends with ϕ_{PPF} of 25%, 50%, and 75%, the bulk tensile modulus increased by a factor of 4.28, 6.21, and 20.9 and the number of attached MC3T3 cells at 4 h

increased by a factor of 1.09, 2.10, and 3.00, respectively. For longer culture time, the difference between the cell numbers on the uncrosslinked and crosslinked PPF/PCL blends with the same ϕ_{PPF} was more significant.

Further, the bulk tensile modulus might not represent the surface stiffness in this heterogeneous system composed of two polymers with distinct mobilities at 37 °C. The composition dependence of cell attachment, spreading, and proliferation on the uncrosslinked samples cannot be simply interpreted using the bulk tensile modulus that did not vary as strongly as the cell behavior did when ϕ_{PPF} was higher than 25%. Surface segregation was expected in the PPF/PCL blends, i.e., the top surface had more mobile PPF chains than the bulk at the physiological condition [183]. In general, the component with a higher mobility tends to appear on the top surface when blended with another component with a lower mobility [183]. Besides the composition gradient along the top surface of the blends, the crystallinity can also vary from the top surface to the bulk in semi-crystalline PCL. It was found that the surface crystallinity of another semi-crystalline polymer polypropylene was much lower than the bulk and such depth-dependent crystallinity can occur over a top layer of 300 nm [184]. At 37 °C, PCL in the blends could melt partially because of the lower values of T_m and crystallinity. The top surface rich of amorphous oligomeric PPF with a T_g much lower than 37 °C was supported by the observation of a tacky surface that was not suitable for AFM characterization.

Extensive characterizations on the top surface (< 500 nm) of semi-crystalline polymers and blends are under investigation in our research group. The surface segregation effect will be correlated with material properties in order to understand the cell behavior on polymer substrates. For the crosslinked blends, PPF was crosslinked into a network with a greatly reduced mobility while PCL still could crystallize in the network although it was weakly suppressed. It is also worthwhile to investigate if PPF appeared more on the disk surface when crosslinked with non-reactive PCL under UV light. The controllability of the behavior of MC3T3 cells using the substrate composition reported in this study will lead us to achieve better understanding on motility, guidance, differentiation, and gene expression of this cell type, which will improve our material design strategies for bone tissue engineering application.

3.5 Conclusions

We employed a comparative model polymer blend system composed of crosslinkable PPF and semi-crystalline PCL to explore the role of chemical crosslinks in determining material properties and cell behavior. Together with crosslinked PPF and PCL, PPF/PCL blends with three compositions in both uncrosslinked and crosslinked forms have been investigated in terms of controllable thermal, rheological, and mechanical properties as well as surface hydrophilicity and morphology. As an oligomeric diluents, amorphous PPF with a T_g of -0.2 °C softened PCL in the uncrosslinked blends but it strengthened

PCL after crosslinking despite that crosslinks suppressed the crystallinity partially. Rheological properties at 140 °C such as G' , G'' , and η_0 decreased with increasing ϕ_{PPF} . T_m for PCL in the uncrosslinked blends decreased slightly while it did not change in the crosslinked blends. Apparent crystallinity decreased with adding more PPF in both uncrosslinked and crosslinked blends. A wide range of mechanical properties has been achieved from a flexible material with a tensile modulus of 38 ± 2.6 MPa for uncrosslinked PPF/PCL ($\phi_{\text{PPF}} = 75\%$) to a stronger and stiffer material with a tensile modulus of 848 ± 62 MPa for crosslinked PPF/PCL ($\phi_{\text{PPF}} = 75\%$) at room temperature. The tensile moduli for the blends and PCL at 37 °C were 40-55% lower. Surface morphology of the crosslinked blends was as smooth as crosslinked PPF until the composition of PCL was high enough (75%) to crystallize substantially. The crosslinked blends were slightly more hydrophilic compared to their uncrosslinked counterparts due to lower crystallinity and smoother surfaces. Weak composition dependence was found for the blends in their capabilities of adsorbing proteins from cell culture medium. All the blends demonstrated excellent cytocompatibility (~100%) when exposed to mouse MC3T3 cells. Distinct cell responses have been found to the PPF/PCL blends in the presence or absence of crosslinks. At the same composition, crosslinked PPF/PCL blends could support MC3T3 cell attachment, spreading, and proliferation significantly better than their uncrosslinked counterparts. In uncrosslinked PPF/PCL blends, cell attachment, spreading, and proliferation were dramatically reduced by blending amorphous PPF with

T_g lower than 37 °C with PCL and such decrease was much more dramatic compared to the composition dependence of the bulk tensile modulus, suggesting that surface segregation occurred and resulted a top layer rich of more mobile PPF and a lower surface stiffness compared to the bulk.

**Chapter 4. Banded Spherulitic Morphology in the Hetero-blends
Consisting of Poly(propylene fumarate) and Poly(ϵ -caprolactone) and
Interaction with MC3T3 Cells**

4.1 Introduction

Understanding cell-biomaterial interactions is among the central tasks in developing biomaterials for tissue engineering applications. Surface chemistry, topology, and stiffness are three major factors in determining cell behavior on biomaterial substrates and scaffolds [6-8]. Extensive studies have been performed on demonstrating the role of morphological features in protein and cellular responses [103-108]. To mimic the natural microenvironments and regulate cell functions, well-defined micro- and nanoscale surface patterns, including grooves, pits, pores, particles, and stochastic roughness, have been fabricated via different techniques such as stereolithography, polymer demixing, electrochemical etching, and molecular self-assembly [103-108]. These morphological features could affect protein adsorption, cell adhesion, cytoskeleton organization, proliferation, differentiation, and gene expression, and *in vivo* tissue formation and organization [103-108].

Spherulites have been found as the characteristic morphological appearance in most crystalline polymers and can be examined by using a polarized optical microscope (POM) [91]. Banded spherulites are spherulites showing regular concentric bands along the radial direction because of polymer lamellar twisting induced by unbalanced surface stress [85-87]. Banded spherulites have been found in numerous crystalline polymers such as polyethylene (PE), polyethylene terephthalate (PET), poly(3-hydroxybutyrate) (PHB), and poly(ϵ -caprolactone) (PCL) [85-87]. PCL is a semi-crystalline and

biodegradable polyester with a glass transition temperature (T_g) of $-60\text{ }^{\circ}\text{C}$ and molecular-weight-dependent melting temperature as well as crystallinity [49]. Banded spherulites have also been observed in miscible blends of PCL with an amorphous polymer species such as polymethacrylates, poly(styrene-*co*-acrylonitrile) (SAN), poly(vinyl chloride) (PVC), and poly(vinyl methyl ether) (PVME), and in copolymers consisting of PCL blocks such as polystyrene-*co*-polybutadiene-*co*-polycaprolactone (SBC), polybutadiene-*co*-polycaprolactone (BC), and polypropylene fumarate-*co*-polycaprolactone (PPF-*co*-PCL), when the PCL composition (ϕ_{PCL}) and crystallization temperatures (T_c) are appropriate [37,88-97].

Processing and fabrication techniques affect the bulk and surface characteristics of the polymer-derived tissue regeneration products, which include thermal and mechanical properties, degradation rate, and topography. These material properties in turn can affect cell-biomaterial interactions and biological performance [98,99]. For semi-crystalline polymers, thermal processing conditions can influence the crystallization and packing of polymer chains and consequently all the properties. For example, poly(L-lactide) (PLLA), a semi-crystalline, biodegradable polymer with a T_g higher than physiological temperature, can become amorphous by rapidly quenching PLLA melt into ice water bath. PLLA samples with controllable crystallinities can also be achieved from isothermal crystallization of the melt at proper temperatures between T_g and T_m .

Because many polymeric biomaterials are semi-crystalline, the effect of crystalline

structure on cellular behavior has been studied, majorly using PLLA. It has been found that spheroid formation, proliferation, and differentiation of hepatocytes were faster on semi-crystalline PLLA substrates while the growth rate of 3T3 fibroblasts was higher on amorphous ones [98]. In another study, pre-osteoblastic MC3T3-E1 cells showed similar density and morphology on both amorphous and crystalline PLLA films and cells were not aligned by the small ridges with a height of ~ 30 nm and a width of ~ 2 μ m formed by the lamellae which radiated from the center of spherulites on crystalline PLLA [99].

Oligomeric, amorphous PPF is an injectable, biodegradable polyester which can be crosslinked into scaffolds with excellent biocompatibility and mechanical strength for bone tissue engineering applications [37]. PPF has been copolymerized with PCL diol to form a series of PPF-*co*-PCL multi-block copolymers [37]. Photo-crosslinked PPF-*co*-PCL networks have a wide range of physical properties to satisfy diverse tissue engineering applications [37]. Because of PCL lamellar twisting along the radial direction during crystal growth induced by the strong surface stress and impeded crystal growth rate, banded patterns have been found in the PCL spherulites when PPF-*co*-PCL ($\phi_{\text{PCL}} = 87\%$) crystallizes at room temperature [37].

Recently we prepared both uncrosslinked and photo-crosslinked forms of binary polymer blends consisting of PPF and PCL for tuning thermal, mechanical, and rheological properties by using physical network associated by crystalline domains and chemical network connected by crosslinks [148]. Mouse MC3T3 cells and rat primary

aortic smooth muscle cells (SMCs) showed distinct cell attachment, spreading, and proliferation to PPF/PCL polymer substrates in the presence or absence of crosslinks. The surfaces of crosslinked PPF/PCL blends became rougher when ϕ_{PCL} increased because the crystalline structure of PCL induced topological heterogeneity. Banded spherulite was not observed on the uncrosslinked PPF/PCL blend films with thicknesses of 160-310 μm cast from solutions and crystallized at room temperature. Nevertheless, because this spherulitic structure has already been found in pure PCL and PPF-*co*-PCL thin films under certain conditions, we hypothesize that it should appear on the films of PPF/PCL blends with proper blend ratio, film thickness, and crystallization condition.

The aim of this work is not only to characterize the thermal properties, crystallization behaviors, and banded spherulitic patterns of these isothermally crystallized hetero-blends of two polymeric biomaterials, PPF and PCL, but also to explore how these crystallization-induced surface morphologies influence surface hydrophilicity, protein adsorption, MC3T3 cell attachment, spreading, and proliferation.

4.2 Materials and Methods

4.2.1 Materials

The molecular weights of PPF and PCL were determined by using an integrated gel permeation chromatography (GPC) system (PL-GPC20, Polymer Laboratories) at room temperature. Tetrahydrofuran (THF) was used as the eluent with a flow rate of 1.0

mL/min and standard and monodisperse polystyrene samples (Polymer Laboratories) were used for calibration. PPF was synthesized via the method reported previously and had a number-average molecular weight (M_n) of 2000 g/mol and a weight-average molecular weight (M_w) of 2800 g/mol [36]. PCL was purchased from Aldrich Co. (Milwaukee, WI) with a nominal molecular weight of 80,000 g/mol and had M_n of 98,000 g/mol and M_w of 144,000 g/mol.

4.2.2 Preparation of Spin-coated PPF/PCL Films

PPF/PCL blends with ϕ_{PCL} of 25%, 50%, 75%, and 100% were prepared by dissolving PPF and PCL in methylene chloride (CH_2Cl_2). Different polymer concentrations, 1.0 g polymer in 5, 7.5, 10, 20, and 50 mL CH_2Cl_2 , were used to obtain various film thicknesses. Then 500 μ L polymer solution was transferred onto round glass cover slips with a diameter of 15 mm and thin films were formed at a spin rate of 1000 rpm for 1 min on a single-wafer spin processor (model WS-400B-6NPP, Laurell technologies Corp., North Wales, PA). After being completely dried in a vacuum oven for at least 48 h to remove the residual solvent, the films were melted at 90 °C for 5 min on a heating plate and then quickly transferred to a hot stage to perform isothermal crystallization at a pre-set temperature.

4.2.3 Characterizations of Isothermally Crystallized PPF/PCL Samples

Differential Scanning Calorimetric (DSC) measurements were performed on a Perkin Elmer Diamond differential scanning calorimeter in a nitrogen atmosphere to

characterize the T_m and crystallinity of isothermally crystallized PPF/PCL blends at T_c ranging from 25 to 52 °C. The sample was heated from 25 to 100 °C at a heating rate of 10 °C/min to record the exotherms. A Nikon POM (Optiphot-2, POL) equipped with a Mettler Toledo FP82HT hot stage was used for monitoring spherulite growth and crystalline morphology. Micrographs were taken at appropriate time intervals with a PixeLink camera. The diameter of the growing spherulite was measured from the micrographs using the ImageJ software (National Institutes of Health, Bethesda, MD) for calculating the crystallization rate. Surface morphology of isothermally crystallized PPF/PCL blends was also characterized using a multimode atomic force microscopy (AFM) with a Nanoscope V control system (Veeco Instruments, Santa Barbara, CA). Tapping-mode images were acquired at room temperature with a scan size of $90 \times 90 \mu\text{m}$ at a scan rate of 0.5 Hz. Topography of the surfaces was recorded simultaneously with a standard silicon tapping tip on a beam cantilever and the root-mean-square (rms) roughness was calculated from height images using image software WSxM 4.0 (Nanotec. Electronica S.L.). Scanning electron microscopy (SEM; S-3500, Hitachi Instruments Inc., Tokyo, Japan) was used to characterize the morphology of PPF/PCL ($\phi_{\text{PCL}} = 75\%$) films crystallized at 45 °C. The thicknesses of spin-coated PPF/PCL films were measured using a Veeco Dektak 6M stylus surface profilometer. Step height was obtained from the cross-section scan profile at a crack on the film surface and five scans were performed for calculating thickness.

4.2.4 Contact Angle Measurement and Protein Adsorption

Approximately one drop of 10 μ L DI water (pH = 7.0) was injected onto PPF/PCL thin films at room temperature. Water contact angle measurement was performed after a static time of 30 s on a Ramé-Hart NRC C. A. goniometer (model 100-00-230, Mountain Lakes, NJ). A tangent method was used to calculate the contact angle in degrees. Three films were used and six values were taken for calculating the average value and standard deviation. For protein adsorption, PPF/PCL thin films spin-coated on glass coverslips were immersed for 4 h at 37 °C in α -Minimum Essential Medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sera-Tech, Germany) and 1% penicillin/streptomycin (Gibco). Then the samples were transferred into 24-well plates (one disk per well) and 600 μ L of phosphate buffer saline (PBS) was used to wash the samples for three times. Ten minutes of gentle agitation was applied and PBS was discarded after each wash. Four hundred microliters of 1% sodium dodecyl sulfate (SDS) solution was put into these wells for 1 h at room temperature. This step was repeated twice and all the SDS solution was collected in a plastic vial. The protein concentration in the collected SDS solution was determined on a micro-plate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA) at 562 nm using a MicroBCA protein assay kit (Pierce, Rockford, IL). Absolute quantification was achieved using a standard curve constructed on albumin solutions in SDS with eight known concentrations.

4.2.5 Cell Attachment and Proliferation

Mouse MC3T3-E1 pre-osteoblastic cells (CRL-2593, ATCC, Manassas, VA) were plated on regular tissue culture flasks with the culture medium in an incubator with 5% CO₂ and 95% relative humidity at 37 °C. Cell culture medium was replaced every 2-3 days and cells were split when they were 80% confluent. MC3T3 cell attachment and proliferation were examined on the spin-coated thin films of PPF/PCL ($\phi_{\text{PCL}} = 75\%$) crystallized at 25, 37, and 50 °C. Thin films spin-coated on the round coverslips with a diameter of 15 mm were sterilized in 70% ethanol for 1 h, washed by PBS, and then completely dried in a vacuum oven. All films were used within two weeks of fabrication and sterilized under UV light for 8 h immediately prior to use. Then the samples were attached on the well bottom of 24-well plates using autoclave-sterilized inert silicon-based vacuum grease (Dow Corning, Midland, MI). MC3T3 cells were seeded onto the polymer films at a density of 1.8×10^4 cells/cm² with 600 μ L of culture medium in each well and the samples were exposed to cells for 4 h, 1, 2, and 4 days. Empty wells and cell-seeded wells with the same density but no exposure to polymer films were used as negative and positive controls, respectively. A colorimetric cell metabolic assay (CellTiter 96 Aqueous One Solution, Promega, Madison, WI) was performed. The number of viable cells was correlated to the UV absorbance at 490 nm measured on incubated assay solution using the micro-plate reader. The attached cells were fixed in 600 μ L of methanol for 3 min, washed twice using PBS, and stained with Harris hematoxylin solution and eosin Y solution. The morphology of attached cells was

observed and recorded by using the Nikon microscope with phase contrast illumination. Cell spreading area was determined and averaged on 25 non-overlapping cells at 4 h and day 1 post-seeding using the ImageJ software.

4.3 Results and Discussion

4.3.1 Thermal Properties of Isothermally Crystallized PPF/PCL Blends

Figure 4.1 shows the DSC curves of PPF/PCL blends with ϕ_{PCL} of 25%, 50%, 75%, and 100% crystallized at various T_c from 25 to 52 °C. There was only one endothermic peak in the heating run for determining melting point (T_m) and the heat of fusion (ΔH_m). The apparent crystallinity χ_c for the blend was calculated using the equation of $\chi_c = \frac{\Delta H_m}{\Delta H_m^c} \times 100\%$ with the known ΔH_m^c value of 135 J/g for completely crystalline PCL [172]. As listed in Table 4.1, both T_m and χ_c of PPF/PCL blends were found to be strongly dependent on ϕ_{PCL} and T_c . As expected, T_m and χ_c generally decreased as the amount of amorphous PPF increased at one specific T_c because of the dilution of crystallizable segments. Both T_m and χ_c increased when T_c increased for one fixed ϕ_{PCL} because more ordered lamella structure could be formed at a slower crystallization rate.

The Hoffman–Weeks approach was adopted to demonstrate the effect of T_c on the T_m of PPF/PCL blends and to obtain the equilibrium melting temperature (T_m^0) by extrapolating the line of T_m versus T_c to the diagonal line $T_m = T_c$ [185]. As shown in

Figure 4.2a, the T_m^0 of PPF/PCL binary mixtures decreased from 72.2 to 60.1, 59.3, and 58.0 °C when ϕ_{PCL} decreased from 100% to 75%, 50%, and 25%, respectively. Polymer blends tend to melt below T_m^0 because of the imperfection and the finite size of crystals [185]. The melting point depression of a crystalline polymer in the blend has been frequently used to estimate the magnitude of the polymer–polymer interactions. The interaction parameter was used to evaluate the miscibility of PPF and PCL which can be commonly examined from the Flory-Huggins equation for melting point depression as follows [186]:

$$\frac{1}{T_m} - \frac{1}{T_m^0} = -\left(\frac{RV_1}{\Delta H_f V_2}\right) \left[\frac{\ln(1-\phi_2)}{n_1} + \left(\frac{1}{n_2} - \frac{1}{n_1}\right)\phi_2 + \chi_{12}\phi_2^2 \right] \quad (1)$$

where V_1 and V_2 are the molar volumes of the repeating unit of the crystalline and amorphous polymers, n_1 and n_2 are the degrees of polymerization of the two components, ΔH_f is the heat of fusion of the fully crystalline polymer, χ_{12} is the Flory-Huggins interaction parameter, R is the gas constant, and ϕ_2 is the volume fraction of the amorphous polymer. By ignoring the entropic term of mixing, the equation can be simplified as follows [187]:

$$\frac{1}{T_m} - \frac{1}{T_m^0} = -\frac{RV_1}{\Delta H_f V_2} \chi_{12}\phi_2^2 \quad (2)$$

B , the interaction energy density of the blends, can be related to χ_{12} as follows [186]:

$$B = \frac{\chi_{12}RT_m}{V_2} \quad (3)$$

Table 4.1. Melting temperature (°C) and crystallinity (%) in parentheses for PPF/PCL blends crystallized at different T_c .

ϕ_{PCL}	$T_c = 25\text{ }^\circ\text{C}$	35 °C	37 °C	40 °C	45 °C	50 °C	52 °C
25%	55.0	55.5	55.6	56.1	56.6	---	---
	(12.0)	(13.4)	(14.0)	(14.2)	(15.1)		
50%	55.9	56.4	56.8	57.0	57.7	58.0	---
	(22.8)	(24.5)	(24.7)	(24.9)	(25.1)	(25.4)	
75%	56.7	57.2	57.4	57.9	58.1	59.0	---
	(30.4)	(31.1)	(31.9)	(32.3)	(33.0)	(34.2)	
100%	58.0	58.4	58.8	59.7	61.2	63.9	64.7
	(36.3)	(37.4)	(37.9)	(40.6)	(42.0)	(45.1)	(46.6)

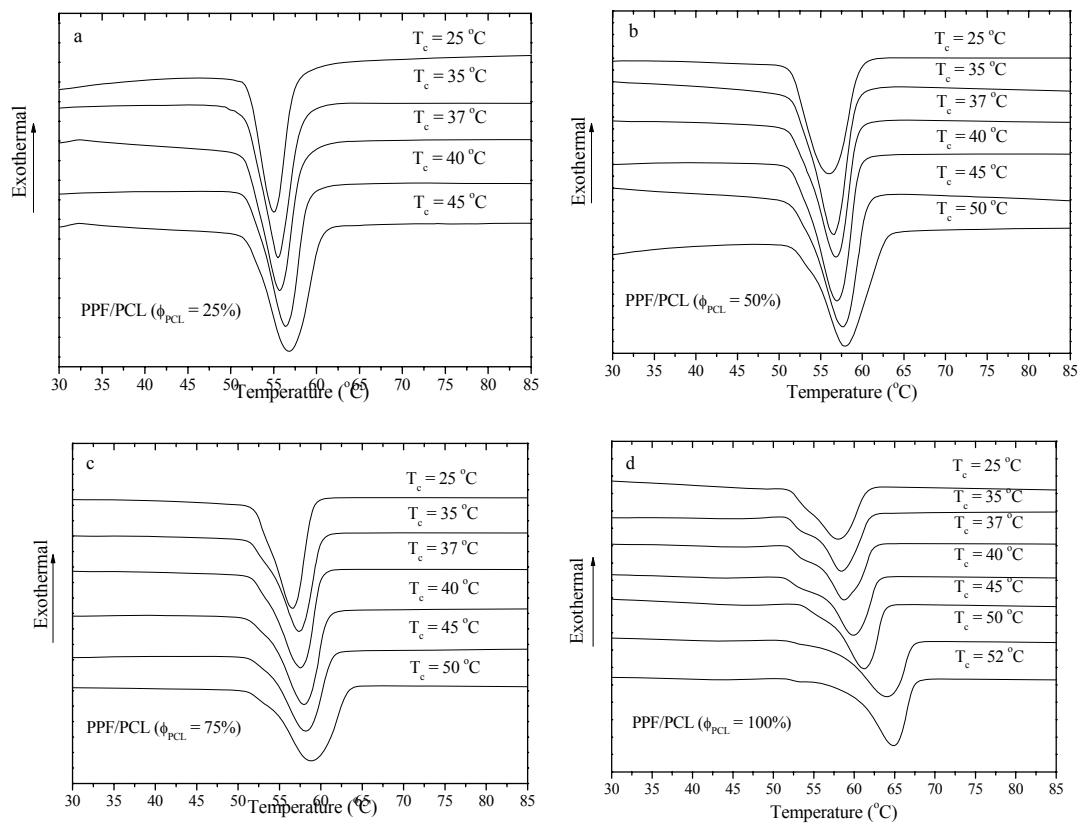


Figure 4.1. DSC curves of PPF/PCL blends with ϕ_{PCL} of 25% (a), 50% (b), 75% (c), and 100% (d) crystallized isothermally at different temperatures from 25 to 52 °C.

By plotting $[(T_m)^{-1} - (T_m^0)^{-1}] \times 10^4$ versus ϕ_2^2 , a fairly consistent line for PPF/PCL blends can be obtained with the slope of 0.44 as shown in Figure 4.2b. With other constants $\Delta H_f = 3690$ cal/mol, $V_{PPF} = 105$ cm³/mol, $V_{PCL} = 99.1$ cm³/mol, $R = 1.98$ cal/mol K, $\rho_{PPF} = 1.29$ g/cm³, and $\rho_{PCL} = 1.14$ g/cm³ obtained from literature [83,88], the $\chi_{PPF-PCL}$ and $B_{PPF-PCL}$ can be calculated as -0.09 and -0.57 cal/cm³ using the above equations. The negative interaction parameter and negative interaction energy density of PPF/PCL blends verified that PPF and PCL were miscible. Moreover, some other miscible polymer blends containing PCL have been reported with negative interaction parameters, such as $\chi_{PCL-SAN} = -0.34$ [93], $\chi_{PCL-PVC} = -0.32$ [188], and $\chi_{PCL-PVME} = -0.11$ [189].

4.3.2 Banded Spherulite Structure

Figure 4.3a shows the spherulite structures of PPF/PCL blends ($\phi_{PCL} = 25\%$, 50%, 75%, and 100%) isothermally crystallized at different T_c . In the polarized optical micrographs, many nucleation centers and small crystallites could be observed for all the blends when the crystallization occurred at room temperature. The size of PCL spherulites increased progressively with T_c . Slightly distorted banded spherulites could be found for pure PCL in a very narrow temperature window between ~ 50 °C and the T_m of ~ 55 °C, because the low-molecular-weight fraction could be melted first to dilute the rest crystallizable PCL chains with longer lengths. No evident extinction crosses could be observed at 45 °C or below because no low-molecular-weight fraction could be melted at

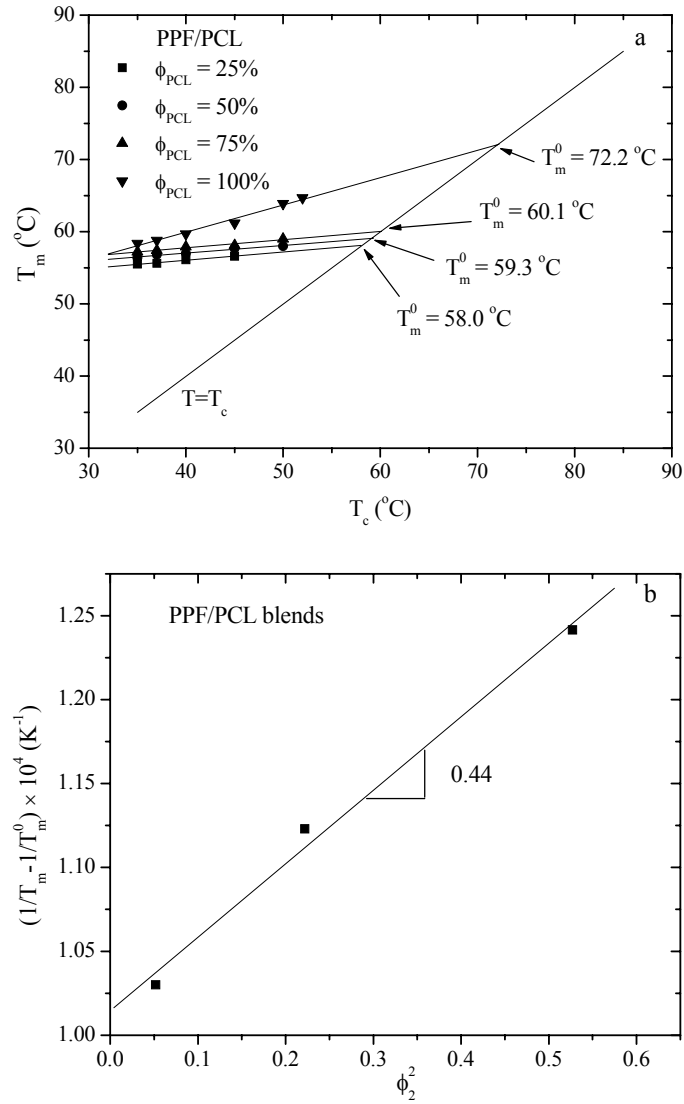


Figure 4.2. (a) Hoffman-Weeks plots of T_m vs. T_c for PPF/PCL blends crystallized isothermally at different temperatures. (b) Plot of the left-hand side of eq. 2 vs. ϕ_2^2 for PPF/PCL blends.

this temperature.

Upon introducing amorphous PPF to form a miscible blend system with PCL, banded spherulites appeared at substantially lower T_c and the ring patterns were more regular (Figure 4.3a). The formation of successive concentric rings on spherulites can be attributed to the lamellar twisting along the radial direction during crystal growth induced by the unbalanced surface stress [87]. Its occurrence implied a high degree of coordination in packing of lamellae within fairly compact structures [91]. Because of the miscibility and intimate contact between PPF and PCL, the amorphous component, PPF, could be easily trapped between the crystalline lamellar of PCL, thus causing the formation of banded spherulites in PPF/PCL blends. When the composition of amorphous PPF in the blends was as high as 75%, coarse, discontinuous, and less clear interference appeared. The boundaries between spherulites formed at high T_c were closely connected as straight lines as the result of slow crystallization and few nucleation sites of PCL [89].

Figure 4.3b demonstrates the composition dependence of the band width or the average width of the bright bands in the extinction rings, which characterizes spherulitic patterns with peculiar periodicity. The band width increased with the increasing ϕ_{PCL} in the PPF/PCL blends. For a fixed ϕ_{PCL} , the band width increased with T_c in a narrow temperature range from 37 to 50 °C. Among all the samples, the banded spherulites of pure PCL had the largest band width of ~15 μm when it was crystallized at 52 °C.

4.3.3 Crystallization Kinetics

Figure 4.4a shows the spherulite growth from the melts of PPF/PCL blends with ϕ_{PCL} of 25%, 50%, 75%, and 100% in the isothermal crystallization at 45 °C. The spherulite growth was radial and the number of nucleation sites decreased when T_c was increased or the composition of amorphous PPF increased. As shown in Figure 4.4b, the spherulite diameter was plotted against the crystallization time for the PPF/PCL blends crystallized at 45 °C. The diameter of the PCL spherulites increased linearly with the crystallization time. The slope of the fitted line, indicating the crystallization rate, decreased gradually from 3.37 $\mu\text{m}/\text{min}$ for pure PCL to 1.67, 1.21, and 0.72 $\mu\text{m}/\text{min}$ after adding 25%, 50%, and 75% PPF, respectively.

The isothermal crystallization rates of PCL spherulites were investigated for all the PPF/PCL blends at different T_c from 25 to 52 °C, as shown in Figure 4.4c. The crystallization temperature dependence of spherulite growth rate for the PPF/PCL blends with ϕ_{PCL} of 25%, 50%, and 75% was similar to that for pure PCL. When T_c was increased, the crystallization rate of PPF/PCL blend all decreased monotonically, regardless of composition. At the same T_c , the crystallization rate was greatly suppressed by increasing the composition of amorphous PPF. The dilution of the crystallizable component could affect not only the free energy necessary for forming a critical nucleus on the crystal surface but also the transport process of crystallizable polymer chains to the growing front of crystal [93]. Similarly, other amorphous polymers such as SAN, PVME, and PVC were also reported to impede the PCL crystallization [93,189,190].

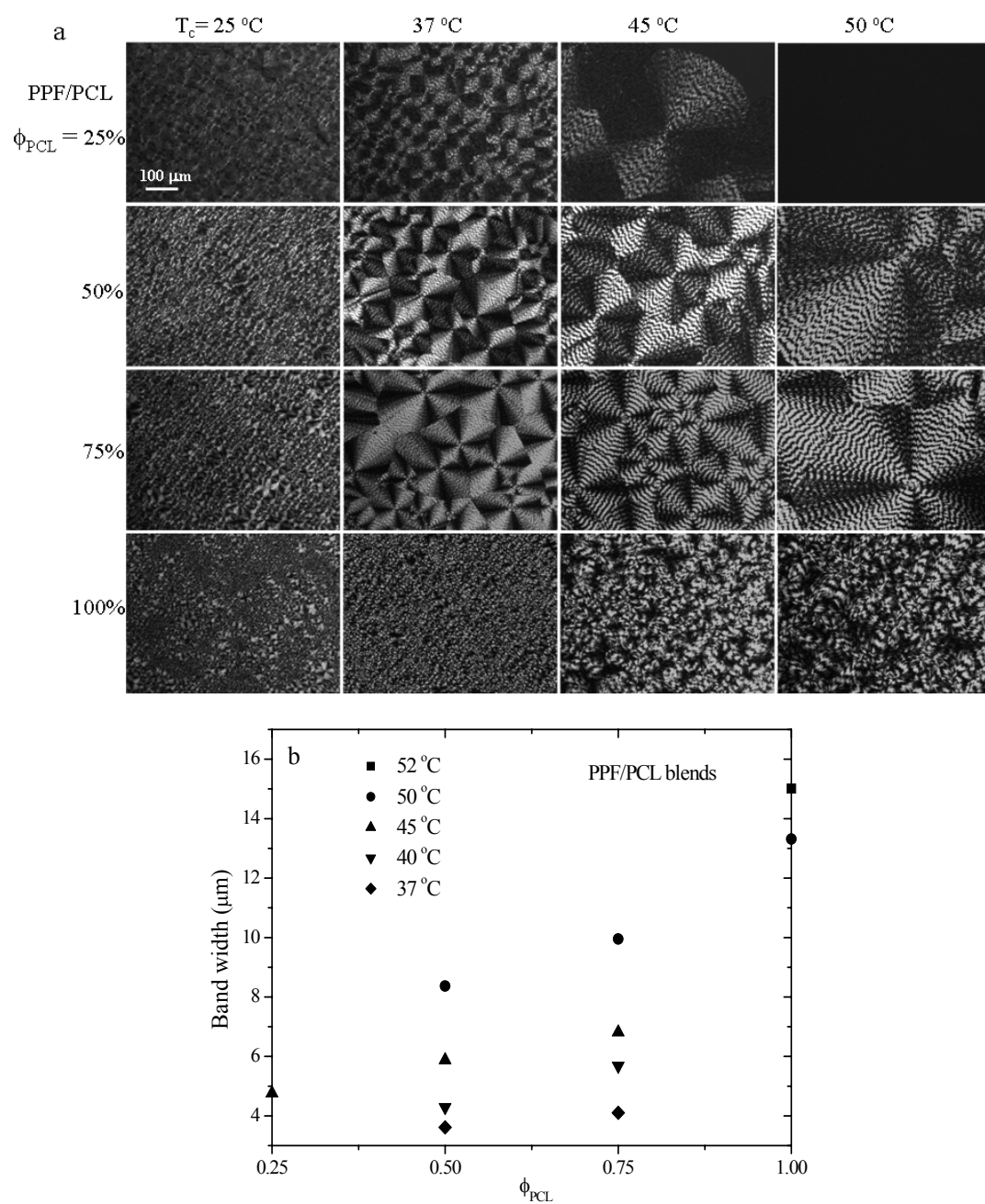


Figure 4.3. Polarized optical micrographs of the crystalline structures (a) and band width of banded spherulites vs. ϕ_{PCL} (b) for PPF/PCL blends crystallized isothermally at different temperatures.

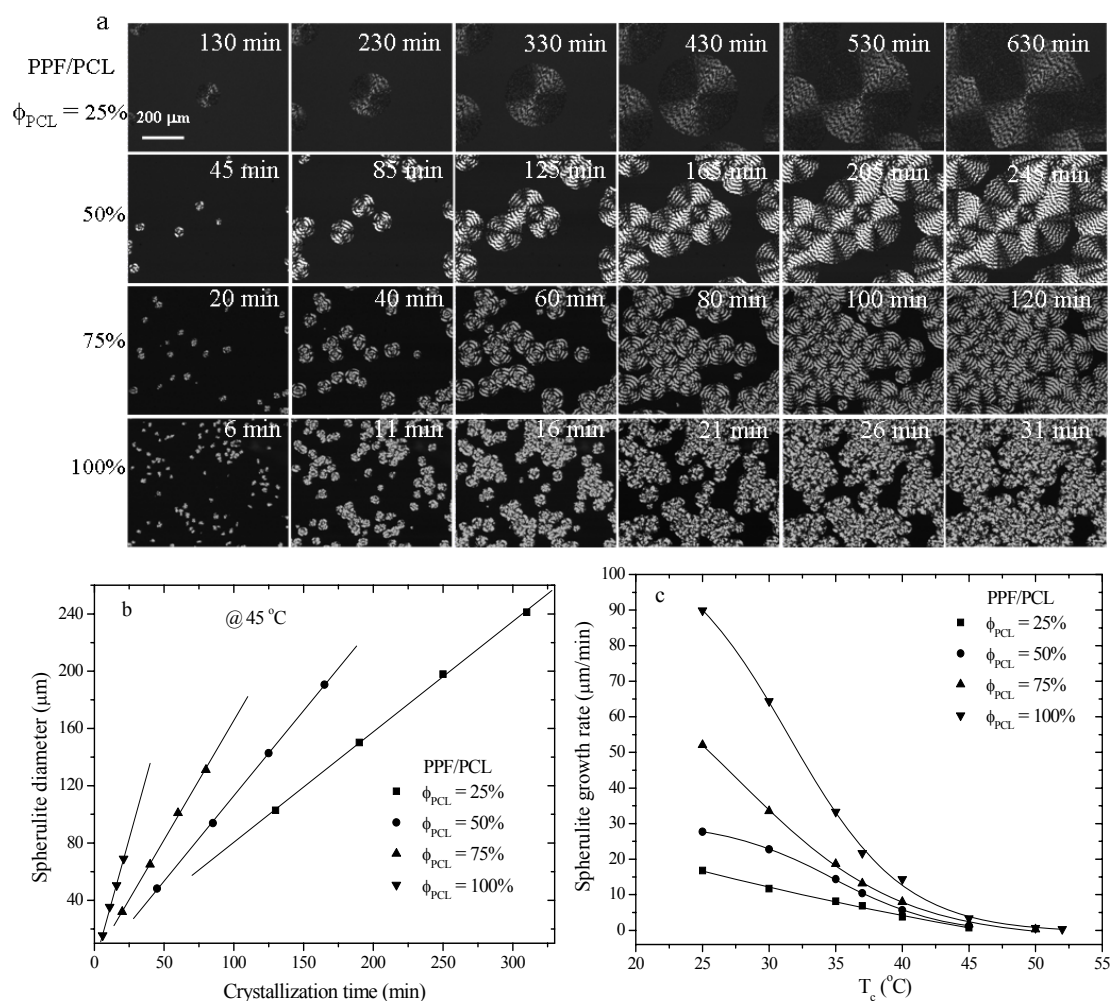


Figure 4.4. (a) Polarized optical micrographs depicting morphological evolution of the spherulites in the PPF/PCL blends crystallized at 45 °C. The time for photographing was marked in the images. (b) The linear relationship between spherulite diameter and crystallization time for PPF/PCL blends crystallized at 45 °C. (c) Spherulite radial growth rate vs. crystallization temperature for PPF/PCL blends.

4.3.4 Surface Morphology of Isothermally Crystallized PPF/PCL ($\phi_{\text{PCL}} = 75\%$) Films

There are several factors to determine the thickness of spin-coated films: the concentration of polymer solution, spin rate, and temperature. In this study, we used the solutions with five different concentrations from 0.02 to 0.2 g/mL to obtain different thicknesses for PPF/PCL ($\phi_{\text{PCL}} = 75\%$) crystallized at 45 °C. The spin-coating was processed at a fixed spin rate of 1000 rpm for 1 min at room temperature. Surface morphology of the films prepared using different solution concentrations was characterized using POM images, as shown in Figure 4.5a. Regular banded spherulites with clear boundaries between white and dark bands can be observed on the PPF/PCL ($\phi_{\text{PCL}} = 75\%$) film spin-coated from polymer solution with a concentration of 0.1 g/mL. The Maltese cross and banded spherulitic structure became less clear and regular on the films prepared with solutions with lower concentrations while coarse and discontinuous interference appeared on the films prepared with more concentrated solutions. Moreover, when the polymer solution concentration decreased from 0.2 to 0.13, 0.1, 0.05, and 0.02 g/mL, the thickness of the spin-coated film decreased from 21.5 ± 0.6 to 8.0 ± 0.2 , 4.6 ± 0.1 , 1.4 ± 0.02 , and 0.36 ± 0.01 μm , respectively. The film thickness as a function of solution concentration is indicated in Figure 4.5b with the curve of the best fit $t = 658.7 c^2 - 29.2 c + 0.9$, where t is the film thickness and c is the solution concentration.

Spin-coated films of PPF/PCL ($\phi_{\text{PCL}} = 75\%$) obtained using the polymer solution of 0.1 g/mL was chosen to examine the effect of T_c on surface morphology and roughness.

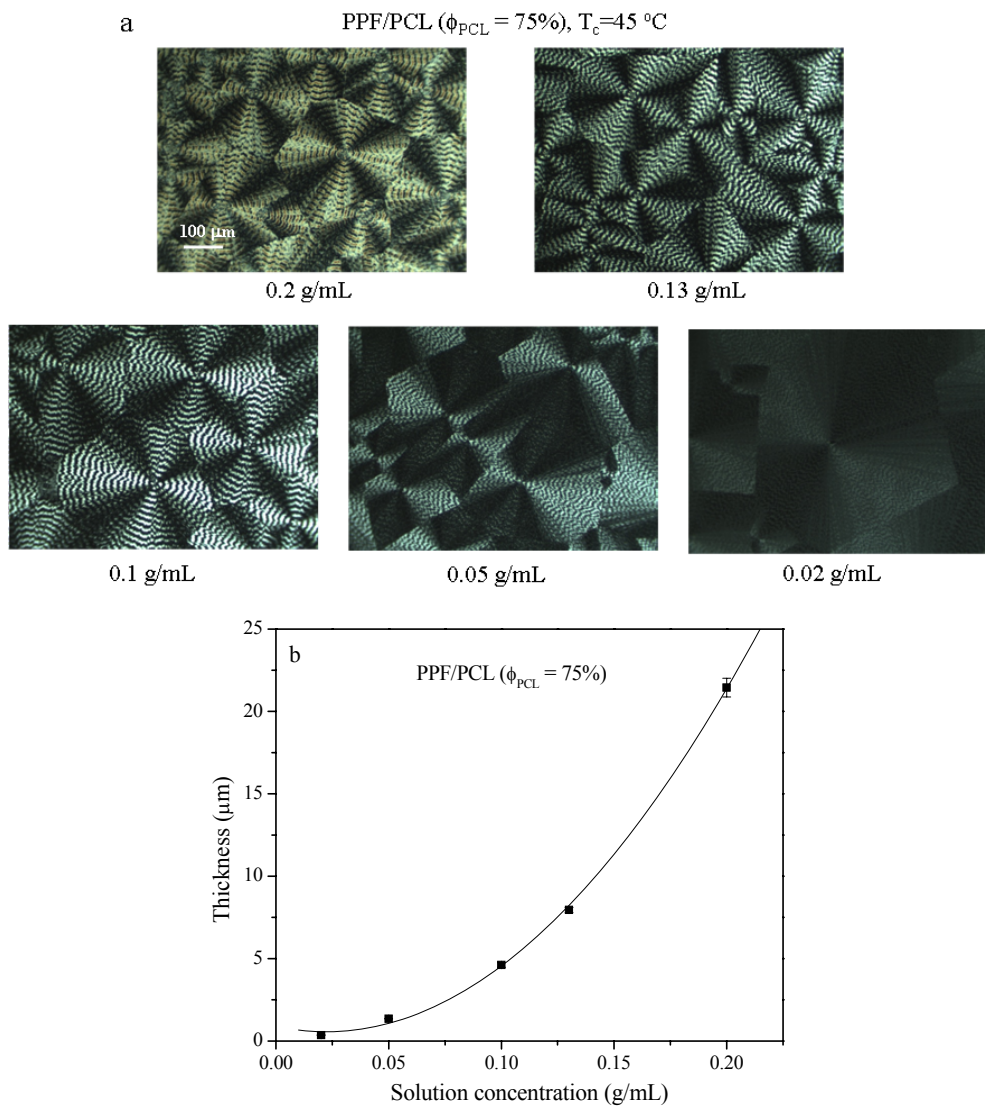


Figure 4.5. (a) Polarized optical micrographs of spin-coated PPF/PCL ($\phi_{\text{PCL}} = 75\%$) films prepared from different concentrations and crystallized isothermally at 45°C . (b) Film thickness of the spin-coated PPF/PCL ($\phi_{\text{PCL}} = 75\%$) as a function of solution concentration.

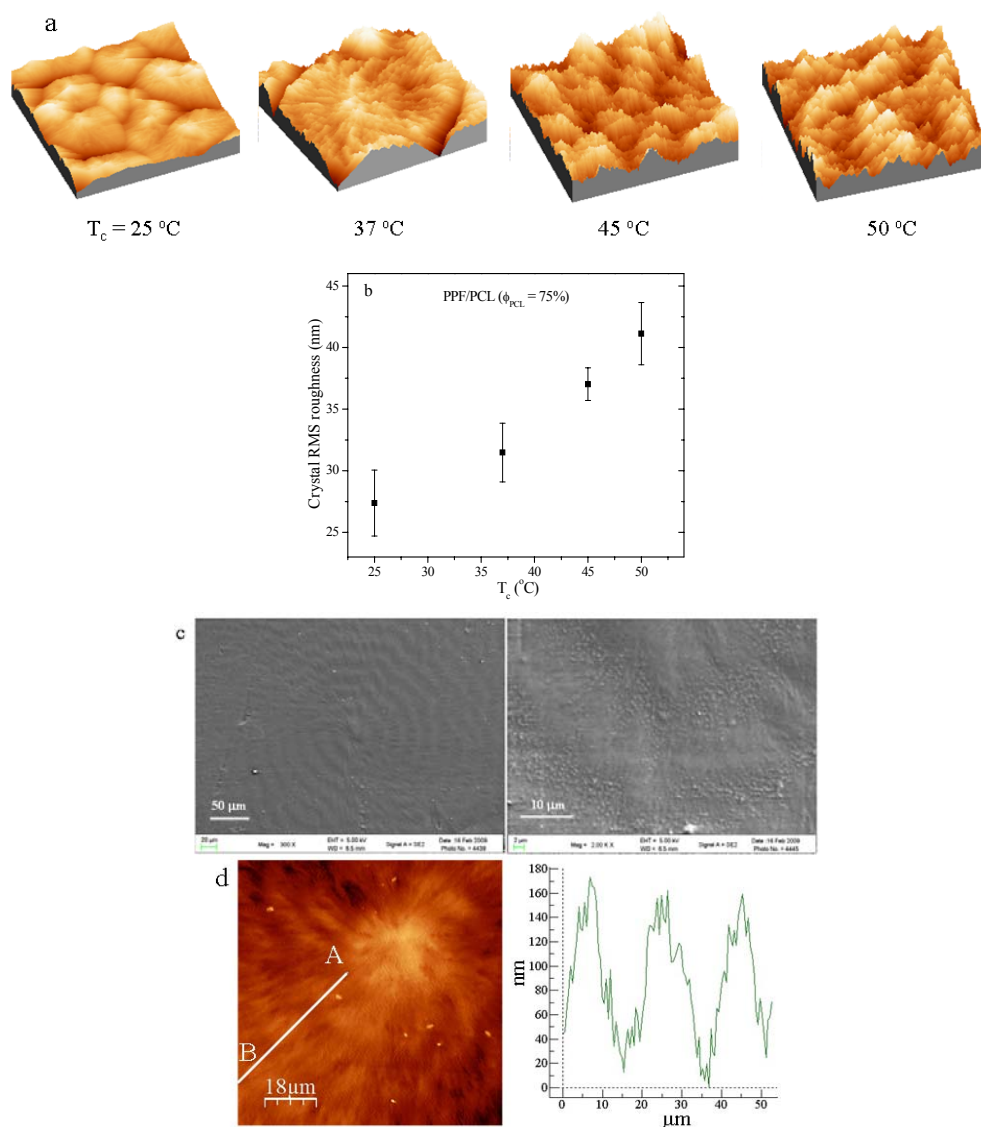


Figure 4.6. AFM images of the spherulitic morphology (a) and root-mean-square roughness of the spherulites (b) on the PPF/PCL ($\phi_{\text{PCL}} = 75\%$) films crystallized isothermally at 25 , 37 , 45 , and 50°C . (c) SEM images of the banded spherulite formed in PPF/PCL ($\phi_{\text{PCL}} = 75\%$) at 45°C with magnifications of $\times 300$ (left) and $\times 2000$ (right). (d) AFM height image of banded spherulite on the PPF/PCL ($\phi_{\text{PCL}} = 75\%$) films crystallized at 45°C and a height profile through A–B.

As shown in the AFM images in Figure 4.6a, there were more nuclei and smaller crystals with a relatively smooth surface when the crystallization occurred at 25 °C. The crystals became much larger and rougher at T_c close to its T_m . Quantified from AFM images, the rms roughness of the crystals (Figure 4.6b) on the thin films of PPF/PCL ($\phi_{PCL} = 75\%$) increased from 27.4 ± 2.7 to 31.5 ± 2.4 , 37.0 ± 1.3 , and 41.1 ± 2.5 nm when T_c was increased from 25 to 37, 45, and 50 °C, respectively. SEM images in Figure 4.6c showed interference rings on one banded spherulite formed on PPF/PCL ($\phi_{PCL} = 75\%$) at 45 °C with different magnifications. Because the band width was much larger than the depth of the band, more evident peak-valley morphology can be observed on the height profile obtained from the 2D AFM image in Figure 4.6d. The height amplitude was ~ 160 nm.

4.3.5 Protein Adsorption and Cell Study of Isothermally Crystallized PPF/PCL ($\phi_{PCL} = 75\%$) Films

Surface topography and surface chemistry such as hydrophilicity are important factors in determining cell-biomaterial interactions and protein adsorption [6-8]. In this study, the effect of crystallites roughness on surface hydrophobicity and protein adsorption was examined using thin films of PPF/PCL ($\phi_{PCL} = 75\%$). As shown in Figure 4.7, the water contact angle of $\sim 89^\circ$ did not vary much for the samples crystallized at different T_c . In contrast, the concentration of serum proteins adsorbed on thin films had dependence on T_c , as shown in Figure 4.7. The protein concentration increased progressively from 17.3 ± 2.0 to 20.6 ± 3.9 , 23.8 ± 3.2 , and 24.6 ± 2.0 $\mu\text{g/mL}$ when T_c

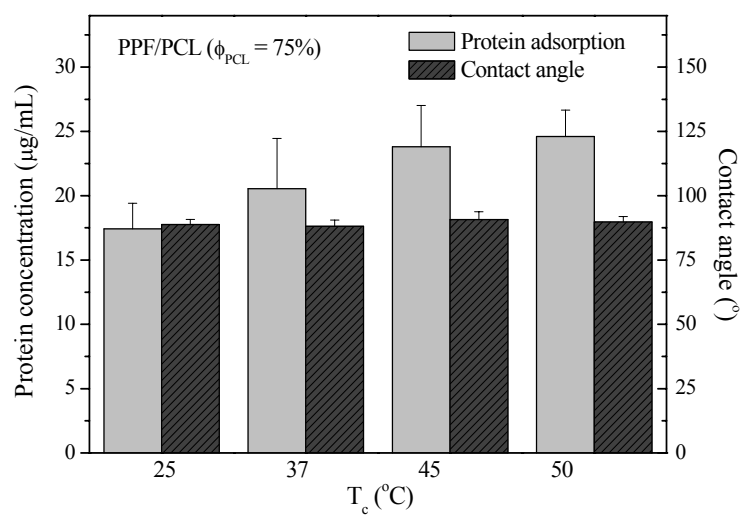


Figure 4.7. Water contact angle and the concentration of proteins adsorbed on the films of PPF/PCL ($\phi_{\text{PCL}} = 75\%$) crystallized at different temperatures.

was increased from 25 to 37, 45, and 50 °C, respectively. The higher capability of adsorbing proteins from cell culture media can be attributed to rougher surface and larger surface area of banded spherulites. Similarly, protein adsorption can also be influenced significantly by micro-machined surface nanostructure [108]. The adsorption of fibrinogen, an extracellular protein, on nanostructured tantalum and titania films fabricated by beam deposition was found to be enhanced with increasing surface roughness [191,192].

Mouse MC3T3 cell behavior were examined on the spin-coated thin films of PPF/PCL ($\phi_{\text{PCL}} = 75\%$) crystallized at 25, 37, and 45 °C. As demonstrated in the previous study [148], PPF/PCL blends did not contain cytotoxic leachable components or degradation products within the period of 4 days for cell studies. Cell spreading and attachment on polymer substrates were crucial for cell motility, proliferation, and tissue organization when these polymers are considered for biomedical applications [193]. At 4 h post-seeding, only a few cells with a rounded phenotype appeared in the images for all the three samples, as demonstrated in Figure 4.8a. Although surface roughness varied from 27.4 to 41.1 nm when T_c was changed from 25 to 45 °C, it did not affect the average spreading area of attached MC3T3 cells (Figure 4.8b). The cell area varied little from 886 ± 202 to 873 ± 266 , and $899 \pm 270 \mu\text{m}^2$ at 4 h, from 1192 ± 331 to 1208 ± 329 , $1260 \pm 245 \mu\text{m}^2$ at day 1 on the films when T_c was increased from 25 to 37 and 45 °C, respectively.

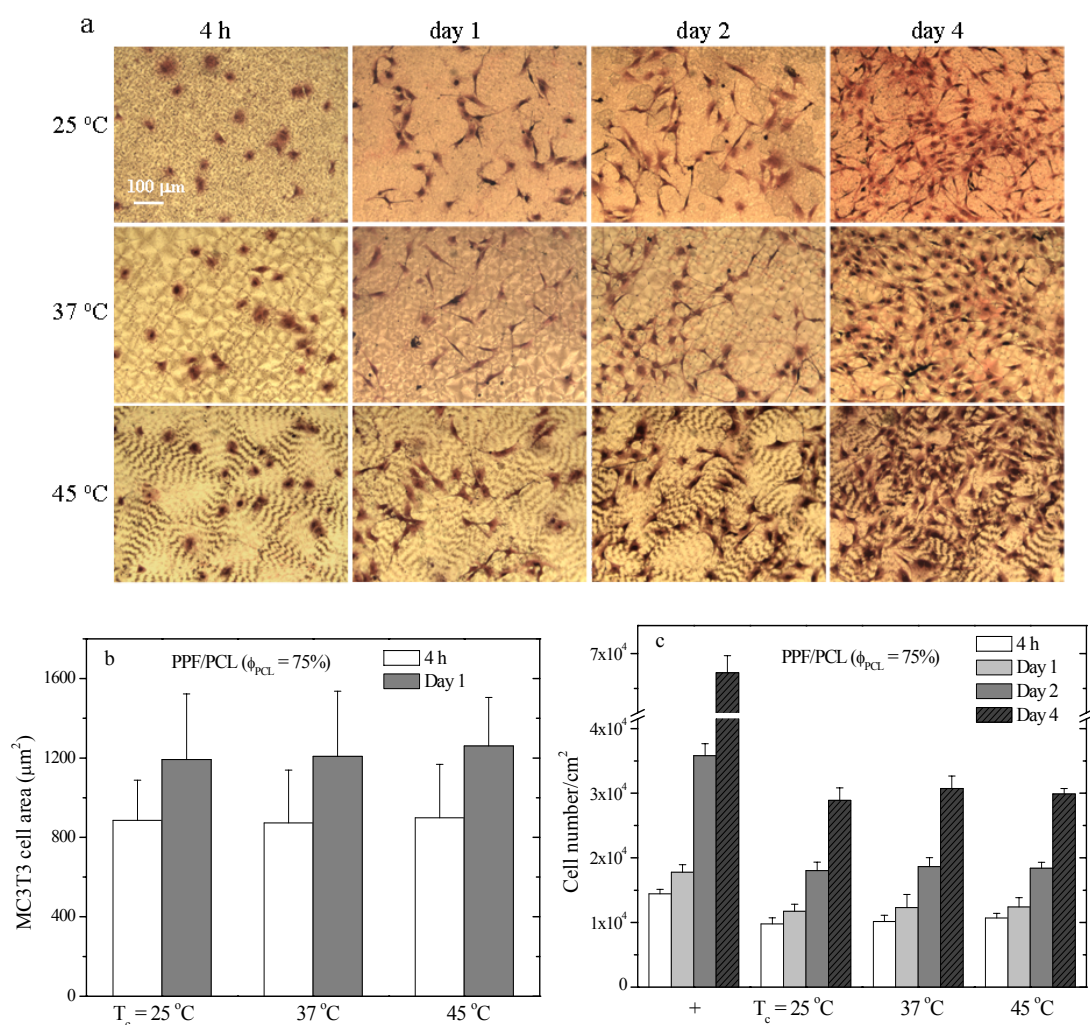


Figure 4.8. Phase-contrast images (a), spreading area (b), and the numbers (c) of MC3T3 cells on the PPF/PCL ($\phi_{PCL} = 75\%$) films crystallized at 25, 37, and 45 °C after culturing for 4 h, 1, 2, and 4 days. The scale bar of 100 μm is applicable for all the images in (a). Cell area in (b) was examined at 4 h and day 1 only. The positive control in (c) is tissue culture polystyrene.

The phase-contrast images of MC3T3 cells on the films of PPF/PCL ($\phi_{\text{PCL}} = 75\%$) at 4 h, days 1, 2, and 4 and their corresponding cell numbers are demonstrated in Figure 4.8a,c. MC3T3 cell number increased with culturing time up to 4 days for all the samples and there was no significant difference in the cell number among the films with different morphologies and roughnesses obtained by varying T_c . Microfabricated substrates with nano- and micro-grooves have been used to align cells through the “contact guidance” effect, which could also influence cell differentiation, gene/protein expression, and *in vivo* tissue development and organization. However, for the crystallization-induced peak-valley morphology formed on the isothermally crystallized films of PPF/PCL ($\phi_{\text{PCL}} = 75\%$), no clear cell elongation or alignment can be observed on the banded spherulites. We speculate that the reasons are low height of the peak compared to the band width and lack of uniform peak-valley structures.

4.4 Conclusions

We have characterized thermal properties, crystallization kinetics, morphology, hydrophilicity of spin-coated thin films of PPF/PCL blends with different compositions isothermally crystallized at different temperatures, and further performed preliminary cell studies on these thin films. Amorphous PPF component could not only decrease the melting point and crystallinity but also impede the crystallization rate of PCL because of the dilution effect. By using the result of the equilibrium melting point depression, the

interaction parameter and interaction energy density of PPF/PCL blends were calculated as -0.09 and -0.57 cal/cm^3 , indicating that PPF and PCL were miscible. Because of the lamellar twisting along the radial direction in crystal growth, banded spherulitic patterns were observed on spin-coated thin films of the PPF/PCL blends at appropriate T_c and ϕ_{PCL} . The film thickness and surface morphology were demonstrated to be strongly dependent on the concentration of polymer solution. By using SEM and AFM, the typical peak-valley morphology of banded spherulites with the height amplitude of $\sim 160 \text{ nm}$ have been observed on the thin films of PPF/PCL ($\phi_{\text{PCL}} = 75\%$) prepared by using a polymer solution with a concentration of 0.1 g/mL . As T_c was increased from 25 to $50 \text{ }^\circ\text{C}$, the rms roughness of the crystals on the thin films of PPF/PCL ($\phi_{\text{PCL}} = 75\%$) increased progressively from 27.4 to 41.1 nm . Meanwhile, the concentration of the proteins adsorbed from cell culture medium increased progressively from 17.3 to $24.6 \text{ }\mu\text{g/mL}$ because of increased surface area and roughness induced by banded spherulites. However, MC3T3 cell attachment, spreading, and proliferation were not significantly influenced by the morphology and roughness of banded spherulites compared with other spherulitic surfaces.

Chapter 5. Banded Spherulitic Morphology in the Poly(ϵ -caprolactone)

Homo-blends and Interactions with MC3T3 Cells

5.1 Introduction

Cell-biomaterial interactions are crucial for the tissue engineering applications of the materials. There are three major categories of surface characteristics to determine cell-biomaterial interactions: surface chemistry, morphological features, and surface mechanics [6-8]. In this study, we focus on the effect of surface morphology on cell behavior. Surface morphological features include ordered patterns and random roughness at both the micron and nanometer scales. Many existing techniques such as stereolithography, polymer demixing, electrochemical etching, and molecular self-assembly have enabled fabrication of substrates with controllable topographical features exhibiting short- and long-range orderliness [102]. To mimic *in vivo* microenvironments and regulate cell behaviors, topographical features such as pits, pillars, pores, grooves, islands, and random rough surfaces have been fabricated with various materials [103-108]. Cells could sense, communicate, and respond to the signal from these surface morphological features and consequently *in vitro* biological responses such as cytoskeleton organization, cell migration, proliferation, differentiation, and gene expression, and *in vivo* tissue organization are effectively influenced [103-108].

Banded spherulite, a unique spherulitic structure with concentric bands along the radial direction which can be seen on a polarized optical microscope (POM), has been reported in semi-crystalline polymers [91]. Formation of banded spherulite can be attributed to polymer lamellae twisting induced by unbalanced surface stresses [86,87].

Poly(ϵ -caprolactone) (PCL) is an widely used biodegradable, semi-crystalline polymer with excellent biocompatibility and mechanical properties [36]. Banded spherulites have been found in pure PCL and miscible blends of PCL with another amorphous component such as poly(vinyl chloride) (PVC), poly(styrene-*co*-acrylonitrile) (SAN), poly(vinyl methyl ether) (PVME), and polymethacrylates, and block copolymers consisting of PCL segments such as polybutadiene-*co*-polycaprolactone (BC), polystyrene-*co*-polybutadiene-*co*-polycaprolactone (SBC), and poly(propylene fumarate-*co*-caprolactone) (PPF-*co*-PCL) when they are crystallized at proper conditions [37,88-97]. For PPF-*co*-PCL with ϕ_{PCL} of 87%, banded patterns were formed when it was crystallized at room temperature because of lamellar twisting in crystal growth, which was induced by the strong surface stress of the PCL lamellae and impeded crystal growth rate [37]. The spin-coated thin films of binary polymer hetero-blends composed of a high-molecular-weight, semi-crystalline PCL and an oligomeric, amorphous PPF have been found by us to show regular banded spherulitic patterns when ϕ_{PCL} was 50% or 75% when they were isothermally crystallized between 45 °C and the melting temperature (T_m).

The methods used for processing polymers used in tissue engineering applications are important to determine material properties, which significantly influence cellular responses, tissue development, and overall medical performance. For semi-crystalline polymers, which are many in biomaterials, their surface can be roughened by spherulites

formed in polymer crystallization [98]. Unlike PCL, another semi-crystalline and biodegradable polymer, poly(L-lactic acid) (PLLA), has been studied in terms of the role of crystallization-induced surface characteristics and cellular responses [99-101]. Because PLLA has a glass transition temperature (T_g) higher than 37 °C, amorphous and semi-crystalline substrates could be prepared using two different methods of quenching and isothermal crystallization and were used to examine the effect of crystallinity on cell behavior [99]. It has been found that hepatocyte adhesion, proliferation and differentiation were enhanced on crystalline PLLA substrates and 3T3 fibroblast cells showed higher proliferation rate on amorphous PLLA samples than on crystalline ones [99-101]. A gradient of polymer crystallinity was also achieved on PLLA films by using different annealing temperatures from 45 to 100 °C and a gradient of surface root-mean-square (rms) roughness (R_{rms}) ranging from 0.5 to 13 nm was formed on the substrate surface [100]. The proliferation rate of pre-osteoblastic MC3T3-E1 cells was greater on the smooth films of PLLA than that on the rough surface and was monotonically dependent on surface roughness [100]. In another study, amorphous poly(D,L-lactic acid) (PDLLA) was blended with semi-crystalline PLLA to achieve a wider gradient of roughness [101]. The surface was smooth on the PDLLA-rich regions while rough on the PLLA-rich regions and a maximum R_{rms} of ~70 nm value was obtained at ϕ_{PLLA} of ~70% [101]. MC3T3 cell adhesion did not vary much when the composition of PDLLA/PLLA blend was different but cell proliferation was faster on the

PDLLA-dominant sample ($\phi_{\text{PDLLA}} = \sim 70\%$) with a lower R_{rms} of 11 nm [101].

Despite of the above-mentioned results, the origin and mechanism of distinct cell responses to crystallization-induced surface characteristics compared with amorphous, smooth polymer substrates is still unclear and these results for cell adhesion and proliferation are often controversial. In this study, we aim to characterize the thermal properties, crystallization kinetics, and banded spherulitic morphologies of isothermally crystallized pure PCL and binary homo-blends containing high- and low-molecular-weight PCL components. Protein adsorption from cell culture media, cell attachment, proliferation, and nucleus localization have also been examined on both hot-compressed disks and spherulites-roughened films using mouse MC3T3 cells to demonstrate the effect of crystallization-induced surface characteristics on cellular behavior.

5.2 Materials and Methods

5.2.1 Materials

Gel permeation chromatography (GPC) was performed at room temperature on an integrated GPC system (PL-GPC20, Polymer Laboratories, Inc.) to determine the molecular weights of PCL samples used in this study. Tetrahydrofuran (THF) was used as the eluent with a flow rate of 1.0 mL/min and standard and monodisperse polystyrene samples (Polymer Laboratories) were used for calibration. PCL80k, PCL2k, and PCL530

were purchased from the Sigma–Aldrich (Milwaukee, WI) with nominal molecular weights of 80000, 2000, 530 g/mol. The number-average molecular weight (M_n) and weight-average molecular weight (M_w) of these three PCL samples measured using GPC were 98,000 and 144,000 g/mol, 3500 and 5200 g/mol, 1080 and 1180 g/mol, respectively.

5.2.2 Sample Preparation

The binary homo-blends of PCL80k/2k and PCL80k/530 with ϕ_{80k} of 25%, 50%, 75%, and 100% were prepared by dissolving 1 g polymer in 10 mL methylene chloride (CH_2Cl_2). Five hundred microliters of the polymer solution was transferred onto a round glass coverslip with a diameter of 15 mm. Then spin coating was conducted on a single wafer spin processor (Laurell technologies Corp., North Wales, PA) at a spin rate of 1000 rpm for 1 min at room temperature. Spin-coated polymer films were melted on a heating plate at 80 °C for 5 min after they were fully dried in a vacuum oven. The melts were quickly transferred onto a hot stage with a pre-set temperature for isothermal crystallization. Smooth sheets of PCL80k/2k ($\phi_{80k} = 75\%$ and 100%) were made by compressing the polymer melts between two clean glass plates. After complete crystallization at room temperature, the polymer sheets were peeled off from the glass plates and cut into round disks with a diameter of 15 mm.

5.2.3 Characterizations

After the binary homo-blends of PCL80k/2k and PCL80k/530 were completely

crystallized at temperatures from 25 to 52 °C, thermal analysis was performed in a Perkin Elmer Diamond differential scanning calorimeter (DSC) in a dry nitrogen atmosphere to obtain their melting temperatures and crystallinities. All the samples were heated to 100 °C with a heating rate of 10 °C/min to record the exotherms. A polarized-light microscope (Nikon Optiphot-2, POL) equipped with a Mettler Toledo FP82HT hot stage and a PixeLink digital camera was used to observe the isothermal crystallization process of all the samples. Growing spherulites were recorded at appropriate time intervals according to the crystallization rate. The diameter of the growing spherulites and the morphological parameters of banded spherulites were analyzed by use of ImageJ software (National Institutes of Health, Bethesda, MD).

Surface morphologies of both hot-compressed and spin-coated PCL80k/2k blends with ϕ_{80k} of 75% and 100% were characterized at room temperature using a multimode Atomic force microscope (AFM) with a Nanoscope V control system (Veeco Instruments, Santa Barbara, CA). Images over a scan area of $90 \times 90 \mu\text{m}$ were acquired using the tapping mode at a scan rate of 0.5 Hz. Surface topography was recorded with a standard silicon tapping tip on a beam cantilever and the root-square-mean (rms) roughness was calculated from the height profiles using image software WSxM 4.0 (Nanotec. Electronica S.L.). The thicknesses of the spin-coated films of both PCL80k/2k and PCL80k/530 blends with ϕ_{80k} of 25%, 50%, 75%, and 100% were measured using a Veeco Dektak 6M stylus surface profilometer. The step height was obtained from a cross

scan at a crack formed using a blade. Five scans were performed and averaged for calculating the film thickness.

5.2.4 Protein Adsorption

Hot-compressed disks and isothermally crystallized spin-coated thin films of PCL80k/2k blends with ϕ_{80k} of 75% and 100% were immersed for 4 h at 37 °C in α -Minimum Essential Medium (α -MEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sera-Tech, Germany) and 1% penicillin/streptomycin (Gibco), which was used for culturing MC3T3 cells. Then the polymer samples were transferred into 24-well plates (one disk per well) and washed with 600 μ L of phosphate buffer saline (PBS) for three times. Ten minutes of gentle agitation was applied and PBS was discarded after each wash. Four hundred microliters of 1% sodium dodecyl sulfate (SDS) solution was put into these wells for 1 h at room temperature. This step was repeated twice and all the SDS solution was collected in a plastic vial. The concentration of adsorbed serum proteins in the collected SDS solution was determined on a microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA) at 562 nm using a MicroBCA protein assay kit (Pierce, Rockford, IL). Albumin supplied in the kit was used to prepare solutions with known concentrations for constructing a standard curve.

5.2.5 *In Vitro* Cytocompatibility, Cell Attachment and Proliferation

Mouse pre-osteoblastic MC3T3-E1 cells (CRL-2593, ATCC, Manassas, VA) were used to evaluate the cytocompatibility of PCL80k and its homo-blend with PCL2k (ϕ_{80k} =

75%). MC3T3 cells were cultured *in vitro* in the culture media mentioned in Section 2.4. After plating, cell suspension was incubated in an incubator with 5% CO₂ and 95% relative humidity at 37 °C. Cell culture medium was replaced every two or three days and cells were split when they were 80% confluent.

Prior to cell studies, polymer samples were sterilized in 70% ethanol solution for 1 h and completely dried in vacuum. The cytotoxicity evaluation was then performed by culturing MC3T3 cells at a density of 2×10^4 cells/cm² in a 24-well plate with 1 mL of primary medium exposed to polymer samples in the trans-wells for 1, 2, and 4 days. Wells seeded with cells at the same density but without polymer samples were used as positive control while empty wells were negative control. A colorimetric cell metabolic assay (CellTiter 96 Aqueous One Solution, Promega, Madison, WI) was performed in each well to obtain the number of viable cells by correlating to the UV absorbance of the solution at 490 nm measured on a microplate reader. Cell viability was then quantified by normalizing the cell number in the sample well to the positive value.

MC3T3 cell attachment and proliferation were examined on PCL80k and its homo-blend with PCL2k ($\phi_{80k} = 75\%$). Sterilized hot-compressed disks and thin films spin-coated on the round coverslips were attached onto the well bottom of 24-well plates using autoclaved inert silicon-based vacuum grease (Dow Corning, Midland, MI). Cells were seeded onto the polymer samples at a density of 1.8×10^4 cells/cm². After 4 h, 1, 2, 4 days, cell numbers were obtained using the same method mentioned in the last

paragraph. Attached cells were fixed in 600 μL of 4% paraformaldehyde (PFA) solution for 10 min, washed twice with PBS, and permeabilized by 0.2% Triton X-100. Then the cells were stained using rhodamine-phalloidin (RP) for 1 h at 37 °C and 4,6-diamidino-2-phenylindole (DAPI) at room temperature for photographing on an Axiovert 25 Zeiss light microscope (Carl Zeiss, Germany)..

5.2.6 Statistical Analysis

Student's *t*-test was performed to assess the statistical significance ($p < 0.05$) of the differences between results.

5.3 Results and Discussion

5.3.1 Effect of Crystallization Temperature on Thermal Properties

DSC measurements were performed to determine the T_m and the heat of fusion (ΔH_m) of the PCL samples after they were fully crystallized at different crystallization temperatures (T_c) ranging from 25 to 52 °C. The crystallinity χ_c was calculated using the equation of $\chi_c = \frac{\Delta H_m}{\Delta H_m^c} \times 100\%$, where $\Delta H_m^c = 135 \text{ J/g}$ for completely crystalline PCL [172]. PCL2k and PCL530 used in this study had two arms ending with hydroxyl groups because they were synthesized by ring-opening polymerization of ϵ -caprolactone initiated by diethylene glycol. Thus there were two or more melting peaks for PCL2k and PCL530 while there was only one endothermic peak for PCL80k initiated by a molecule with a

single hydroxyl group [78].

As shown in the DSC curves in Figures 5.1 and 5.2, both T_m and ΔH_m increased progressively when PCL80k and its homo-blends with PCL2k or PCL530 were crystallized at a higher T_c because PCL chains had longer time to reorganize into a more ordered lamella structure. T_m and χ_c obtained from the DSC curves are listed in Tables 5.1 and 5.2. T_m was 56.0 °C for pure PCL80k. At a fixed T_c , the T_m of the blend increased with ϕ_{80k} because PCL2k and PCL530 had much lower T_m values of 44.5 and 5.5 °C, respectively. With increasing the composition of PCL2k in the homo-blends, the secondary melting peak at a lower temperature eventually appeared. When T_c and ϕ_{80k} were fixed, PCL80k/2k blends had higher T_m and χ_c compared with PCL80k/530 blends because PCL2k had much higher T_m and χ_c than PCL530. The χ_c of PCL80k/2k blend was the highest when ϕ_{80k} was around 25% and 50% while the χ_c of PCL80k/530 blend decreased monotonically with adding more lower- χ_c component PCL530. Consequently, both T_m and χ_c could be modulated by using the blend composition and T_c .

As demonstrated in Figure 5.3, the Hoffman–Weeks plot was used to obtain the equilibrium melting temperature (T_m^0) by extrapolating the line of T_m versus T_c to the line $T_m = T_c$ [185]. The effect of T_c on the T_m of PCL and its homo-blends with PCL2k and PCL530 can be observed evidently. The T_m^0 of PCL80k/2k binary homo-blend decreased from 72.2 to 68.9, 63.3, and 60.1 °C when the composition of PCL2k increased from 0% to 25%, 50%, and 75%, respectively. Correspondingly, the T_m^0 of PCL80k/530 binary

Table 5.1. Melting temperature (°C) and crystallinity (%) in parentheses for PCL80k/2k blends at different T_c .

T_c	25 °C	35 °C	37 °C	40 °C	45 °C	50 °C	52 °C
$\phi_{80k} = 25\%$	53.5	54.7	55.0	56.0	56.8		
	(34.7)	(35.1)	(35.8)	(37.2)	(43.1)	---	---
$\phi_{80k} = 50\%$	55.3	55.9	56.3	57.3	58.0	60.1	
	(40.3)	(41.2)	(41.5)	(42.3)	(44.3)	(44.6)	---
$\phi_{80k} = 75\%$	57.0	57.8	57.9	58.6	60.5	62.8	
	(39.8)	(40.9)	(41.3)	(42.9)	(43.9)	(46.0)	---
$\phi_{80k} = 100\%$	58.0	58.4	58.8	59.7	61.2	63.9	64.7
	(36.3)	(37.4)	(37.9)	(40.6)	(42.0)	(45.1)	(46.6)

Table 5.2. Melting temperature (°C) and crystallinity (%) in parentheses for PCL80k/530blends at different T_c .

T_c	25 °C	35 °C	37 °C	40 °C	45 °C	50 °C	52 °C
$\phi_{80k} = 25\%$	54.0	54.5	54.7	54.9	57.0		
	(14.9)	(15.2)	(16.2)	(16.3)	(16.8)	---	---
$\phi_{80k} = 50\%$	54.1	55.6	55.9	56.3	57.9	59.9	
	(23.6)	(27.5)	(27.8)	(28.1)	(28.6)	(29.2)	---
$\phi_{80k} = 75\%$	55.2	56.1	56.7	57.6	58.9	62.8	
	(29.6)	(32.1)	(33.1)	(34.3)	(36.5)	(41.3)	---
$\phi_{80k} = 100\%$	58.0	58.4	58.8	59.7	61.2	63.9	64.7
	(36.3)	(37.4)	(37.9)	(40.6)	(42.0)	(45.1)	(46.6)

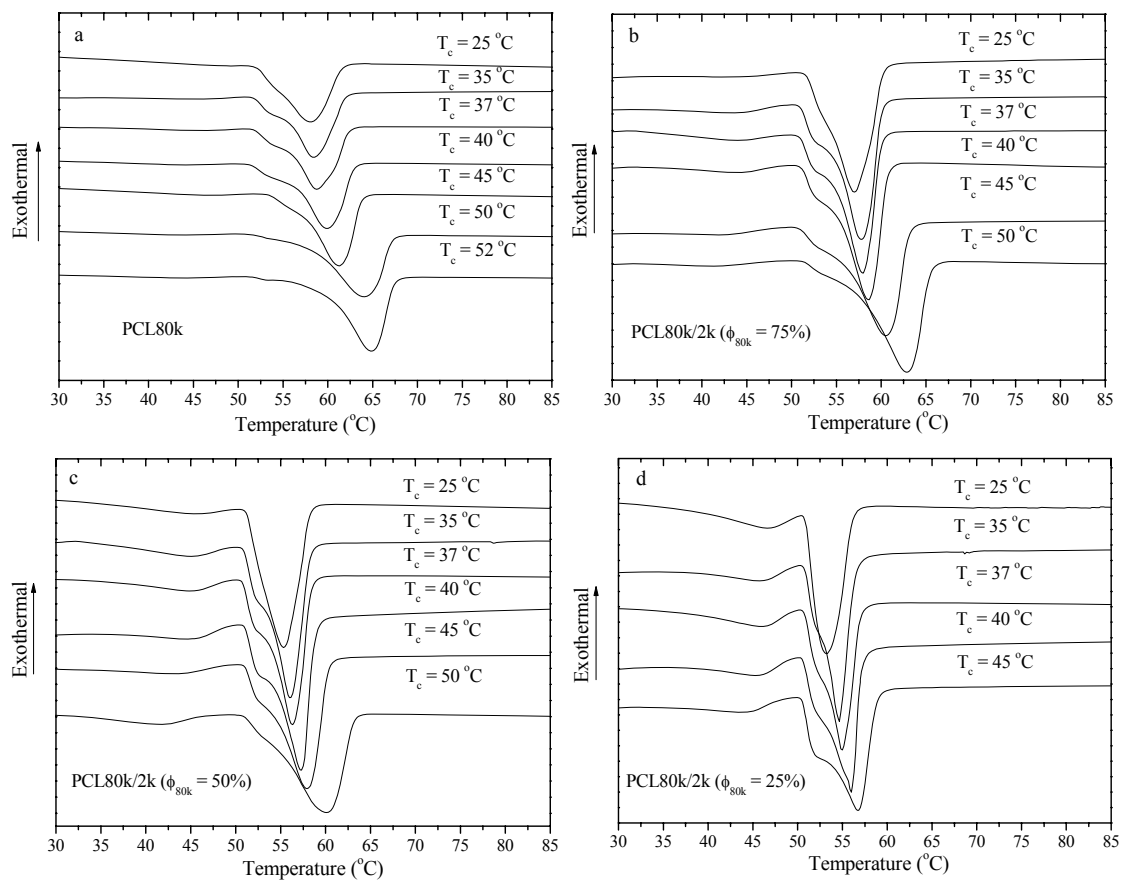


Figure 5.1. DSC curves of PCL80k (a) and PCL80k/2k blends with ϕ_{80k} of 75% (b), 50% (c), and 25% (d) isothermally crystallized at various temperatures from 25 to 52 °C.

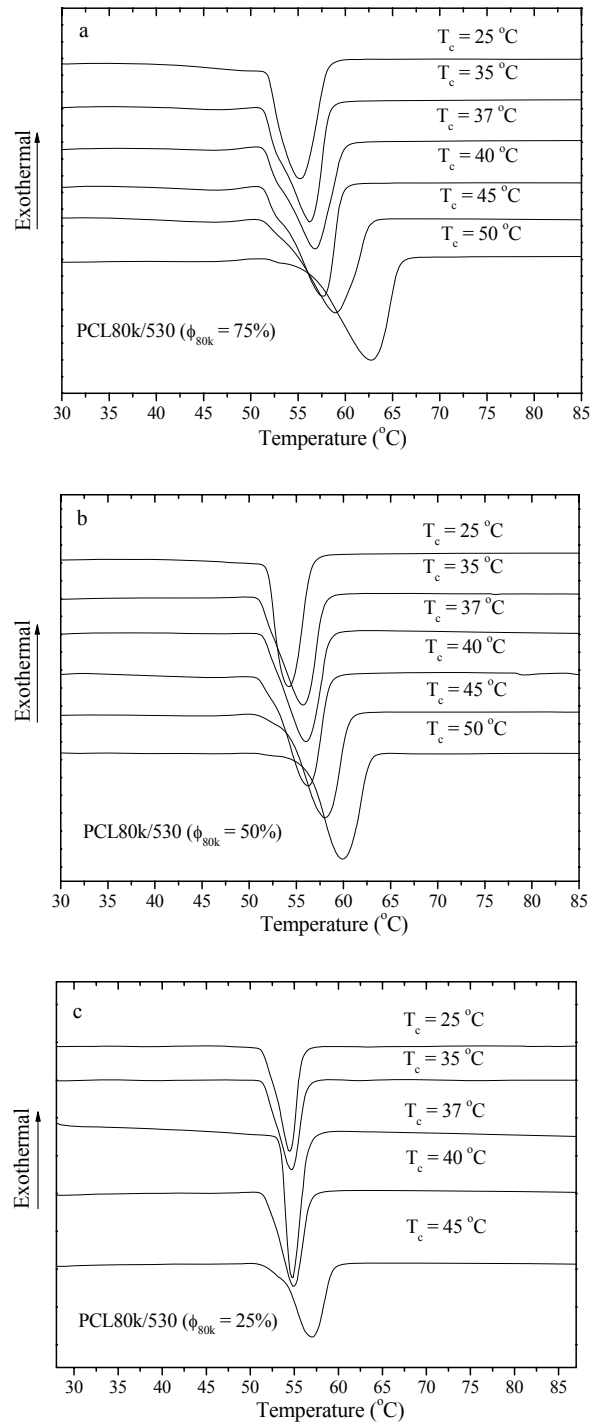


Figure 5.2. DSC curves of PCL80k/530 blends with ϕ_{80k} of 75% (a), 50% (b), and 25% (c) isothermally crystallized at various temperatures from 25 to 50 °C.

blends also decreased from 72.2 to 66.5, 62.5, and 59.3 °C and the value was slightly lower than that of PCL80k/2k blend at the same composition. Polymer blends tend to melt below T_m^0 and the decrease in T_m can be attributed to the finite size and imperfection of crystallites [185].

5.3.2 Spherulitic Morphology

A surface profilometer was used to measure the thicknesses of the spin-coated thin films of PCL80k/2k and PCL80k/530 blends prepared by spin-coating polymer solution with a concentration of 0.1 g/mL. The film thickness decreased from 6.01 ± 0.07 to 5.68 ± 0.08 , 3.98 ± 0.07 , and 2.61 ± 0.04 μm when the composition of PCL2k increased from 0% to 25%, 50%, and 75%, respectively. The same trend was found for PCL80k/530 blends but the thicknesses were lower for the same compositions: 4.84 ± 0.07 , 3.20 ± 0.09 , and 1.27 ± 0.04 μm . The decreased thickness by adding more low-molecular-weight PCL in the binary blends can be attributed to the lower solution viscosity compared to the solution of pure PCL80k with the same concentration. At the same ϕ_{80k} , the spin-coated films of PCL80k/2k blends were thicker than those of PCL80k/530 blends because the viscosity of solid-like PCL2k was much higher than that of liquid-like PCL530 and consequently more polymer mixtures of PCL80k/2k were retained on the round cover slips during spin-coating process [78].

The POM images in Figures 5.4a and 5.5a show the spherulitic morphologies of PCL80k/2k and PCL80k/530 blends with ϕ_{80k} of 25%, 50%, 75%, and 100% when they

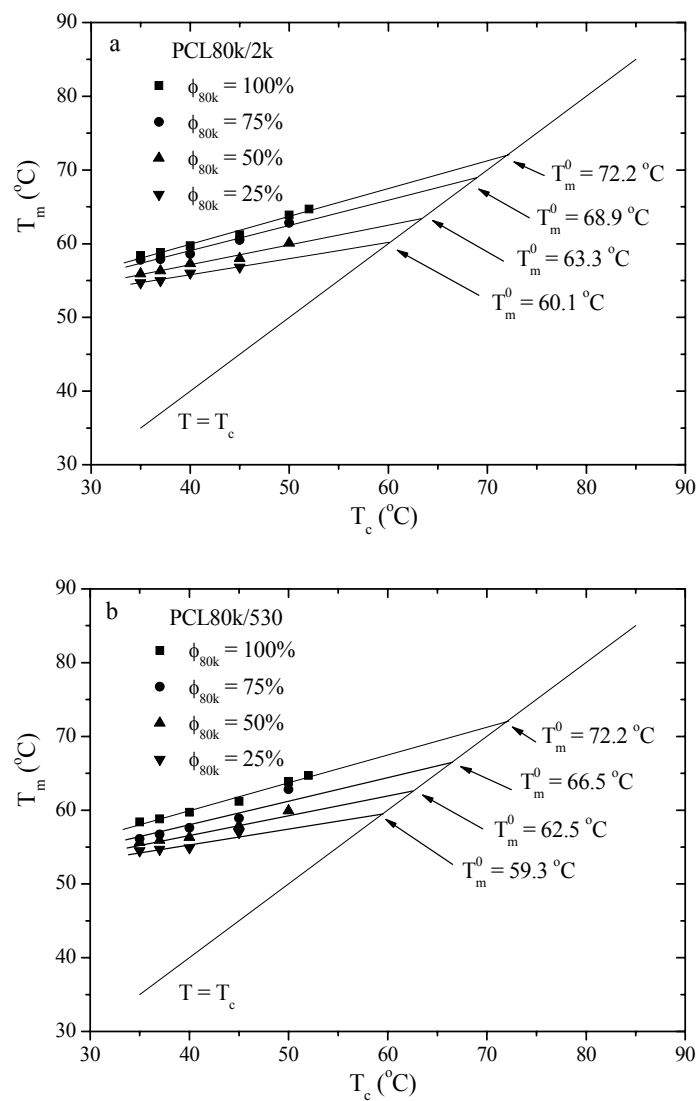


Figure 5.3. Hoffman-Weeks plots of T_m vs. T_c for PCL80k and its blends with PCL2k (a) and PCL530 (b) isothermally crystallized at various temperatures.

were isothermally crystallized at 25, 37, 45, and 50 °C. High degree of nucleation and small crystallites were found at T_c of 25 °C for all the samples. The size of PCL spherulites increased gradually when T_c was increased. Banded spherulites could be formed in pure PCL80k at T_c of ~50 °C and in its blends with PCL2k or PCL530 with ϕ_{80k} of 50% and 75% at T_c of ~45 °C. This unique spherulitic pattern had successive concentric rings. Its occurrence implied a high degree of coordination in packing of lamellar within fairly compact structures [91].

PCL2k and PCL530 with lower T_m in the blends became amorphous at T_c of 45 °C while the main component PCL80k was still able to crystallize with a relatively low rate. Amorphous PCL2k or PCL530 chains were trapped into PCL80k crystalline lamellar structures to help form banded spherulites. When the composition of PCL2k was greater than 50% in the blends, distorted interference rings with more coarse and discontinuous bands appeared. For PCL80k/530 blends with ϕ_{80k} less than 50%, spherulitic structures were no longer clear and the images were dark. At the latter stage of crystallization, spherulites impinged on the neighbors and the boundaries between spherulites became straight because of the effect of low crystallization rate and nucleation of PCL [89].

As one important feature of banded spherulites, band width or the average width of the concentric bright bands was measured from the POM images in Figures 5.4a and 5.5a. The composition dependence of band width is demonstrated in Figures 5.4b and 5.5b. With a similar trend observed in other blends containing PCL [88,89,194], band width

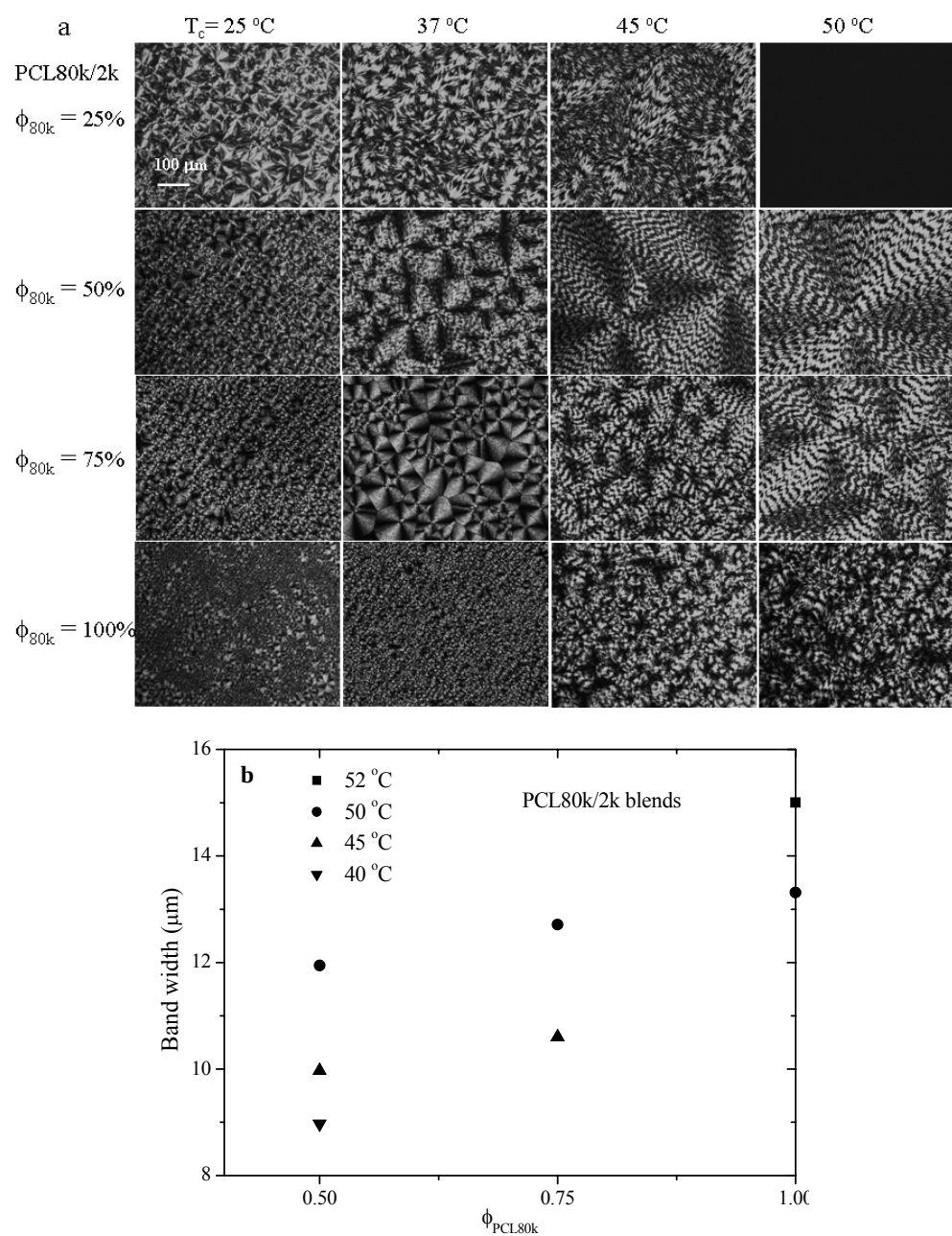


Figure 5.4. Polarized optical micrographs of the crystalline structures (a) and the band width of banded spherulites (b) formed in PCL80k/2k blends when they were crystallized at various temperatures.

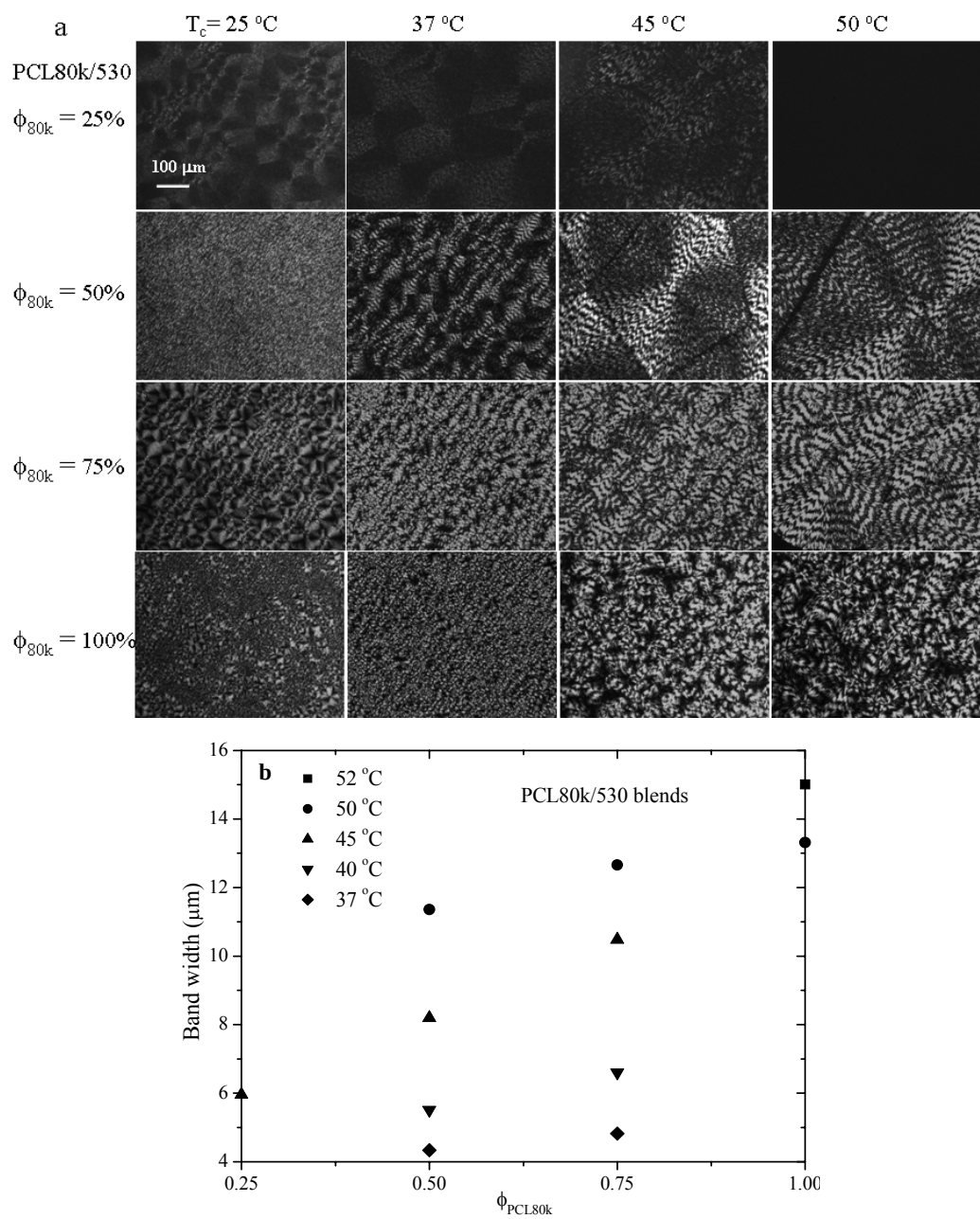


Figure 5.5. Polarized optical micrographs of the crystalline structures (a) and the band width of banded spherulites (b) formed in PCL80k/530 blends when they were crystallized at various temperatures.

increased when ϕ_{80k} was higher in these homo-blends. At the same ϕ_{80k} , the band width increased with T_c in a narrow temperature range from 40 to 52 °C.

5.3.3 Crystallization Kinetics

The POM images in Figure 5.6a shows the spherulites growth from the melt during isothermal crystallization at T_c of 25, 37, 45, and 50 °C, exemplified by PCL80k/2k (ϕ_{80k} = 50%). POM images were taken at different time points with proper intervals to demonstrate the morphological evolution. When T_c was higher, the nucleation step became more difficult and there were fewer nucleation centers for crystal growth in the field, thus spherulites was larger. The spherulite growth was radial and the crystallization rate was calculated by measuring the average diameter of growing PCL spherulites over time for all the samples.

As summarized in Figure 5.6b, the dependence of spherulite growth rate on T_c for binary PCL blends was very similar to that for pure PCL80k and the crystallization rate decreased progressively as T_c increased. The spherulite growth rate of PCL80k was increased after blending with PCL2k having a higher crystallinity. In contrast, it was lower in the blends with a lower-crystallinity component PCL530. The crystallinity and crystallization rate of PCL are generally higher when the molecular weight is lower [195]. However, PCL2k and PCL530 are PCL diols and the molecular architecture and hydroxyl end-groups are detrimental to the formation of ordered crystalline structure, especially when the molecular weight is lower. PCL530 with a low T_m of 5.5 °C was essentially an

amorphous component in the studied range of T_c . Similar to other amorphous polymer species blended with high-molecular-weight PCL having substantial crystallinity and T_m higher than 50 °C, PCL530 significantly impeded the crystallization of PCL80k because the dilution of crystallizable polymer chains can influence the free energy of nucleus formation and the transport process of the crystallizable component to the growing front of crystals [93,189,190].

5.3.4 Surface Morphology of the Samples

The spin-coated films and hot-compressed disks of PCL80k and its blend with PCL2k ($\phi_{80k} = 75\%$) were used to examine the effects of processing method and T_c on surface morphology and roughness. It can be observed in the AFM images in Figure 5.7a,e that the hot-compressed disks of PCL80k and PCL80k/2k ($\phi_{80k} = 75\%$) were flat without spherulitic structures. According to the POM images in Figure 5.4a, there were many small crystallites on the spin-coated thin films when the crystallization occurred at 25 °C and they became larger when T_c was increased. Consistent with POM images, large spherulites with sharp boundaries could be observed in the AFM images in Figure 5.7b,f and regular banded spherulites with roughened surfaces appeared when T_c was close to the T_m of the sample as shown in Figure 5.7c,d,g.

More evidently, the height profiles in Figure 5.8b,d obtained from the 2D AFM images (Figure 5.8a,c) of these two samples demonstrated that the peak-valley morphology occurred at high T_c , which resulted in rougher surfaces with larger surface

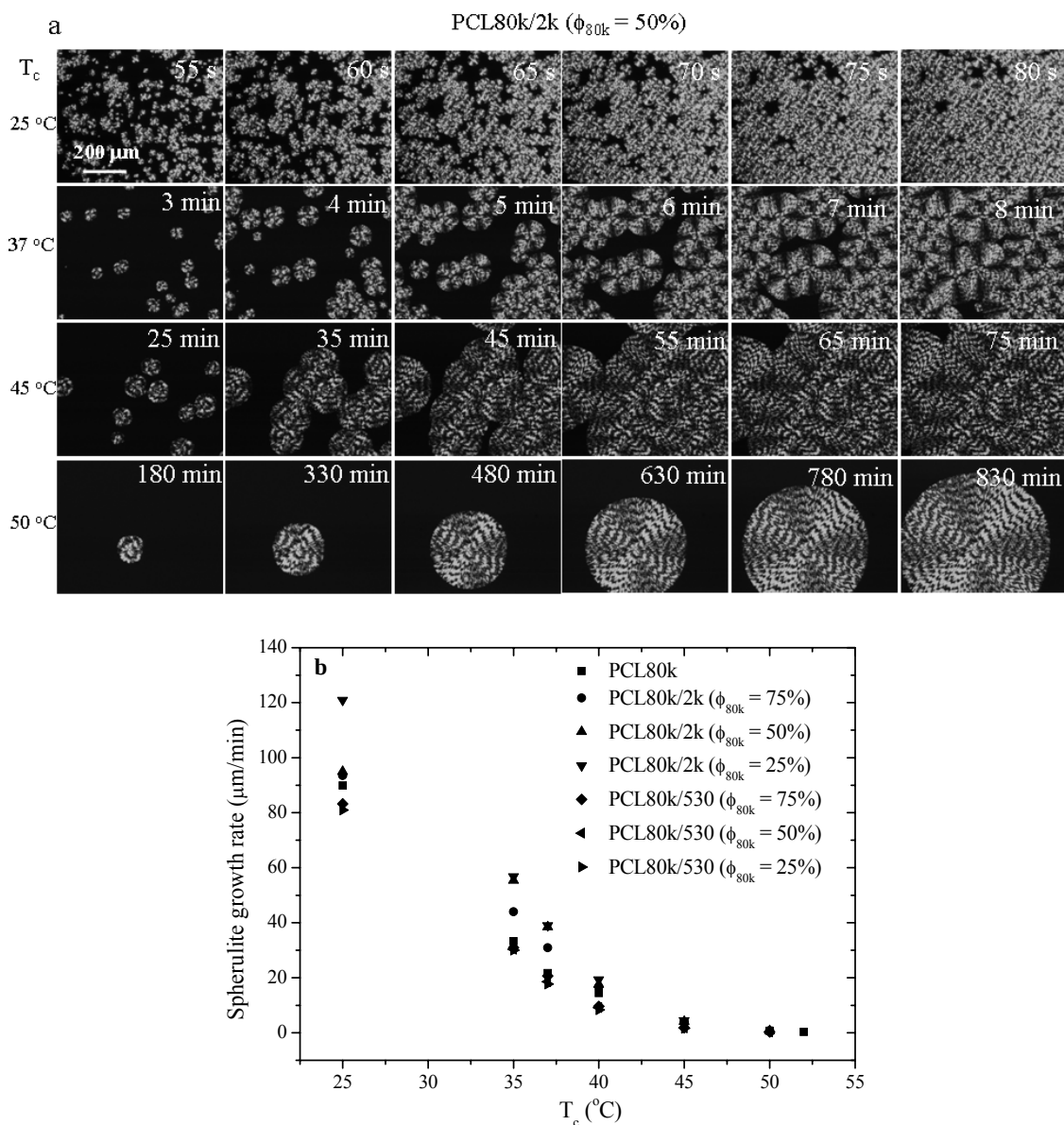


Figure 5.6. Crystallization kinetics. (a) Polarized optical micrographs depicting the morphological evolution of the spherulites in PCL80k/2k ($\phi_{80k} = 50\%$) at different crystallization temperatures (T_c). The time for photographing was indicated. (b) Spherulite radial growth rate at different T_c for PCL80k and its blends with PCL2k and PCL530.

areas. PCL80k crystallized at 52 °C had a larger height amplitude of ~350 nm than that of ~170 nm for PCL80k/2k ($\phi_{80k} = 75\%$) crystallized at 50 °C. This difference could lead to different R_{rms} for the banded spherulites. As indicated in Figure 5.8e, the R_{rms} values of hot-compressed disks were 17.6 ± 1.4 and 20.6 ± 1.8 for PCL80k and PCL80k/2k ($\phi_{80k} = 75\%$), respectively. The values were higher for spin-coated thin films and they increased further when T_c was higher because of the formation of banded spherulites. For PCL80k, R_{rms} values were 36.0 ± 2.2 and 72.9 ± 4.8 nm when it was crystallized at 25 °C and 52 °C, respectively. The values for PCL80k/2k ($\phi_{80k} = 75\%$) were lower, 32.8 ± 1.5 and 48.1 ± 3.5 nm when T_c was 25 and 50 °C, respectively.

5.3.5 Protein Adsorption and Cell Behavior on the Samples

When a polymer surface is exposed to cell culture media, protein adsorption is the first step and can occur in seconds. The ability to adsorb serum proteins from culture media is important for later cell responses to the substrate. Surface chemical modification using hydrophilic poly(ethylene glycol) tethered chains can significantly decrease protein adsorption on the surface of a polymer substrate [38]. The adsorption of proteins can also be influenced by surface nanostructure and roughness [108]. In this study, protein adsorption on the polymer samples used in AFM characterization was examined by use of a micro-BCA protein assay kit. As demonstrated in Figure 5.9, the concentration of the proteins adsorbed on the sample surface increased progressively from 18.3 ± 1.0 $\mu\text{g/mL}$ for hot-compressed disk to 19.5 ± 1.1 and 21.8 ± 2.8 $\mu\text{g/mL}$ for PCL80k spin-coated

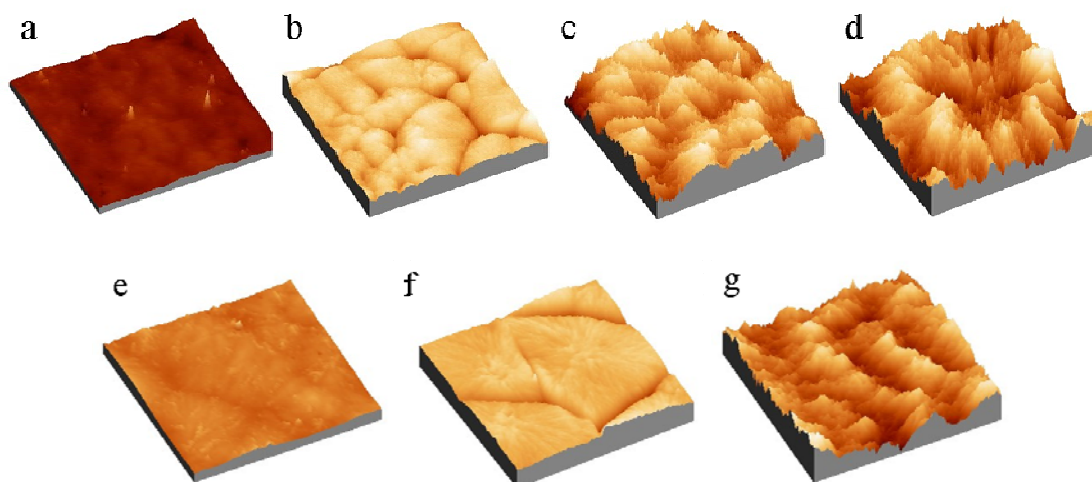


Figure 5.7. AFM images of a hot-compressed disk (a) and spin-coated thin films of PCL80k crystallized at 25 °C (b) and 52 °C (c,d), and a hot-compressed disk (e) and spin-coated thin films of PCL80k/2k ($\phi_{80k} = 75\%$) crystallized at 25 °C (f) and 50 °C (g).

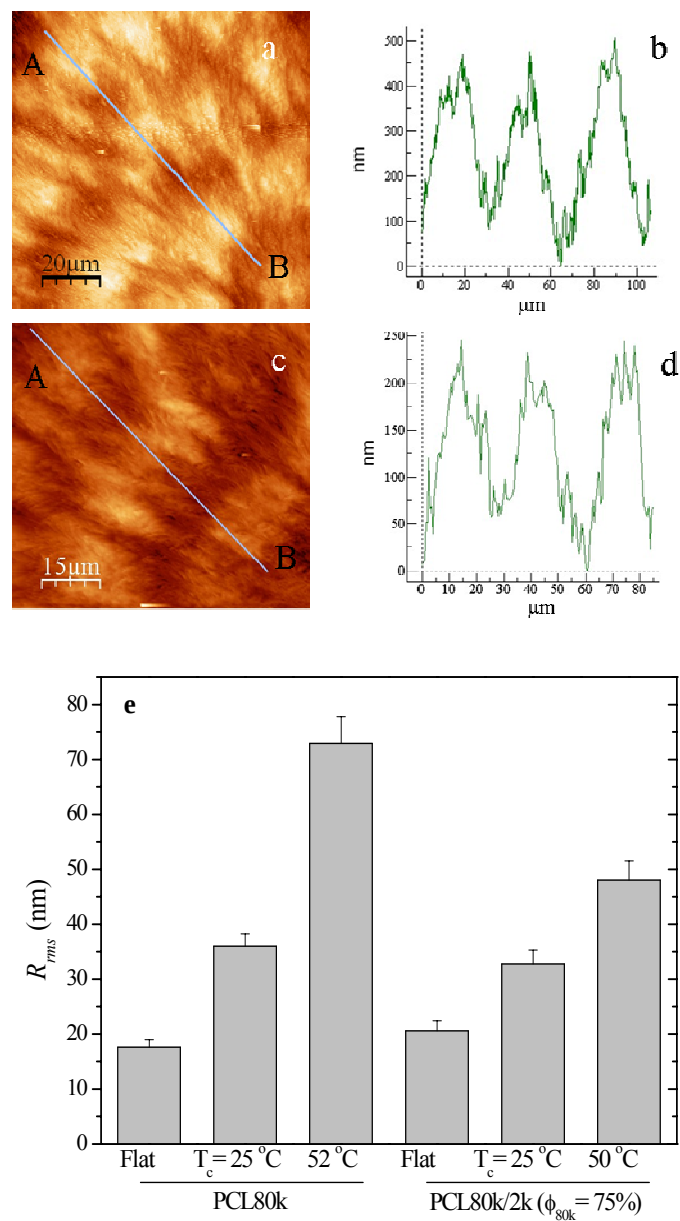


Figure 5.8. AFM height images of banded spherulites obtained on the spin-coated thin films of PCL80k crystallized at 52 °C (a), PCL80k/2k ($\phi_{80k} = 75\%$) crystallized at 50 °C (c). A–B height traces (b, d) and root-mean-square (rms) roughness (R_{rms}) (e).

films crystallized at 37 and 52 °C, respectively. For PCL80k/2k ($\phi_{80k} = 75\%$), the value also increased from $19.7 \pm 1.3 \mu\text{g/mL}$ for hot-compressed disk to 21.6 ± 1.6 and $22.7 \pm 2.0 \mu\text{g/mL}$ for spin-coated films crystallized at 37 and 50 °C, respectively. The enhanced protein adsorption can be attributed to higher surface roughness and area resulted from the formation of banded spherulites, as discussed earlier using AFM images. It has also been found that the adsorption of fibrinogen, an extracellular protein, on nanostructured tantalum and titania films fabricated by use of beam deposition could be enhanced with increasing roughness [191,192].

MC3T3 cell behavior was examined on these two series of polymer samples with varied composition and surface characteristics. Cell viability was found to be close to 100% when the cells were cultured in presence of all the samples for 1, 2, and 4 days. This result indicated that the polymer samples did not contain leachable, toxic components or degradation products within 4 days, during which cell attachment and proliferation were performed. As demonstrated in our earlier study, PCL samples used in this study had very low degradation rate and did not show noticeable degradation in the duration of cell studies.

Cell attachment and spreading on polymer substrates was crucial for cell motility, growth, and tissue formation when polymers are considered for biomedical applications. Cell attachment was monitored at 4 h post-seeding by normalizing the cell numbers on the samples to the number in the positive control. As demonstrated in Figure 5.10a,

normalized MC3T3 cell attachment increased when the substrate was changed from hot-compressed disks with a flat surface to spherulite- and banded spherulite-roughened films for both PCL80k and PCL80k/2k ($\phi_{80k} = 75\%$). In addition, there was a significant difference for cell attachment between PCL80k samples and their PCL80k/2k ($\phi_{80k} = 75\%$) counterparts prepared through the same condition. This difference may be attributed to lower surface stiffness and higher density of hydroxyl end-groups in PCL2k. Represented by the cell numbers at days 1, 2, and 4 in Figure 5.10b, cell proliferation over this period showed a trend similar to that found in cell attachment. Cell numbers were lower on hot-compressed samples compared to the spherulite-roughened samples for both PCL80k and PCL80k/2k ($\phi_{80k} = 75\%$). Also similar to cell attachment, MC3T3 cell proliferation decreased significantly when 25% PCL2k was added in to PCL80k.

MC3T3 cell nucleus localization on banded spherulites was also investigated by combining fluorescence microscopy with transmitted white light microscopy with crossed polarizers [98]. As demonstrated in Figure 5.11a, it is interesting to note that cell nuclei showed preferential location on the banded spherulites at day 4. Percentage of nuclei located in or around the valley of the banded spherulitic structure was analyzed and plotted in Figure 5.11b. The value was higher than 75% for PCL80k crystallized at 52 °C. It decreased to ~65% for PCL80k/2k ($\phi_{80k} = 75\%$) crystallized at 50 °C because of the apparent difference in the feature height amplitude of banded spherulites. We also seeded more cells on the banded spherulites and found the degree of the nucleus preferential

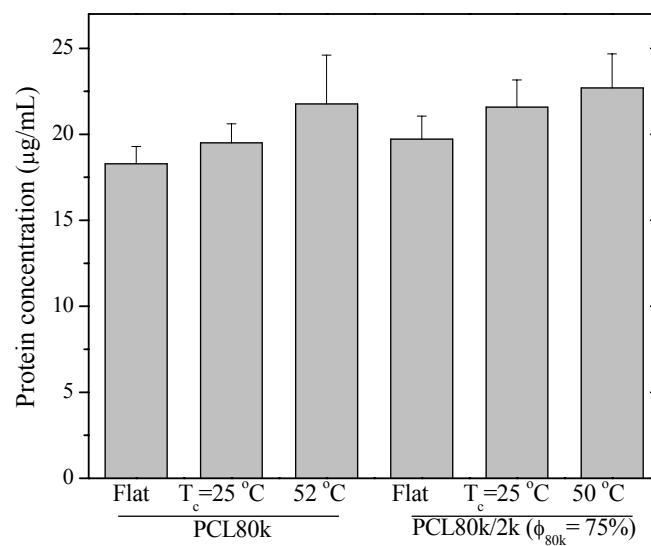


Figure 5.9. Concentration of serum proteins adsorbed on the flat, hot-compressed disks and spin-coated films of PCL80k and PCL80k/2k ($\phi_{80k} = 75\%$) blend isothermally crystallized at different temperatures.

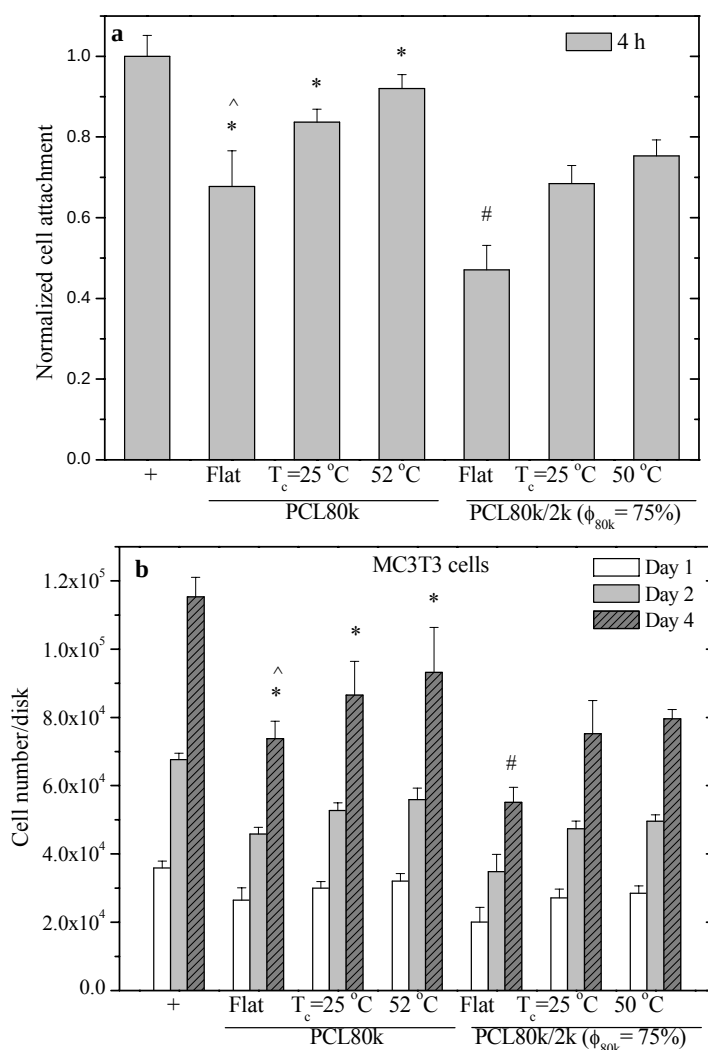


Figure 5.10. Normalized MC3T3 cell attachment at 4 h (a) and number of MC3T3 cells at days 1, 2, and 4 post-seeding (b) on the flat hot-compressed disks and spherulite-roughened films of PCL80k and its blend with PCL2k ($\phi_{80k} = 75\%$), compared with cell-seeded TCPS as the positive (+) control. * $p < 0.05$ relative to the PCL80/2k ($\phi_{80k} = 75\%$) counterparts and TCPS. ^ $p < 0.05$ relative to PCL80k thin films crystallized at 37 and 52 $^\circ\text{C}$. # $p < 0.05$ relative to PCL80/2k ($\phi_{80k} = 75\%$) crystallized at 37 and 50 $^\circ\text{C}$.

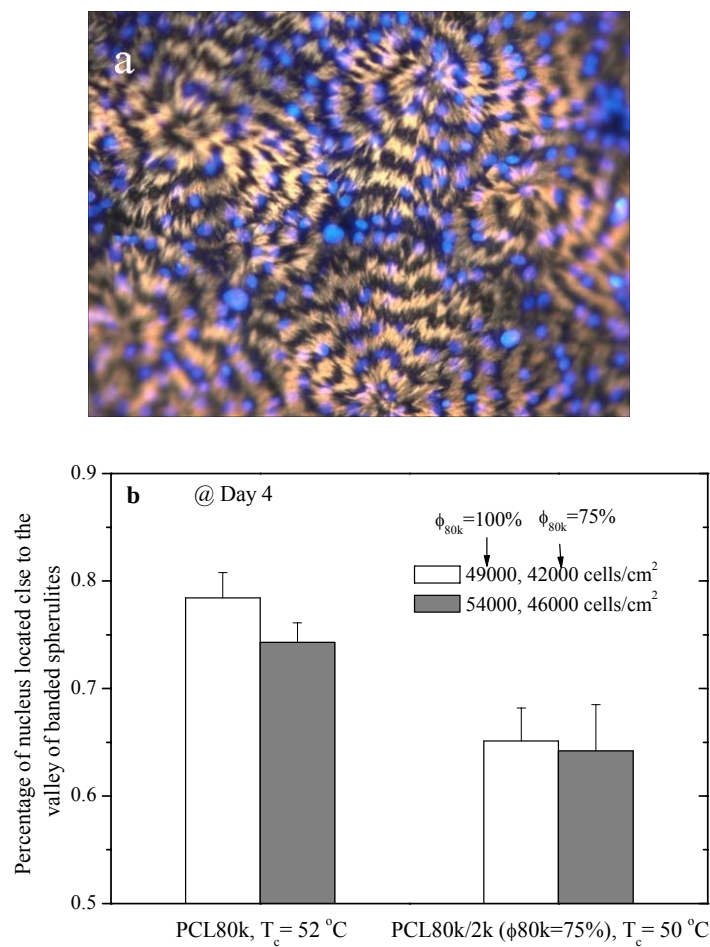


Figure 5.11. (a) MC3T3 cells on PCL80k thin films crystallized at 52 °C after culturing for 4 days demonstrated by merging a DAPI-stained fluorescent image and a POM image together. (b) Percentage of cell nuclei located in or close to the valley of the banded spherulites formed in PCL80k and PCL80k/2k ($\phi_{80k} = 75\%$) when they were crystallized at 52 and 50 °C, respectively. Two different cell densities were used.

location dropped slightly for both samples, as shown in Figure 5.11b. Further discussion on cell-material interactions is elaborated in Section 5.4.

5.4 Further Discussion

The difference in cell behaviors on pure PCL80k and its homo-blend with PCL2k suggested that the low-molecular-weight component could influence surface morphology and physicochemical properties significantly at 37 °C because it had higher density of hydroxyl end-groups with more mobile characteristics. The tensile moduli of PCL80k and PCL80k/2k ($\phi_{80k} = 75\%$) at 25 °C were 361 ± 23 and 327 ± 11 MPa, respectively [196]. The values decreased to 241 ± 10.2 and 215 ± 8.5 MPa at 37 °C. Their compressive moduli were ~ 155 MPa at room temperature. Their bulk mechanical moduli were very similar; however, they might not represent surface stiffness. Surface segregation could occur in PCL80k/2k blends and played an important role in determining cell fate. In general, the component with a higher mobility is liable to appear on the top surface when it is blended with the other component with a lower mobility [183]. At physiological temperature, low-molecular-weight PCL2k with a higher chain mobility and a lower T_m of 44.5 °C in the blend could melt partially. The top surface of PCL80k/2k ($\phi_{80k} = 75\%$) could be rich of amorphous oligomeric PCL chains with a T_g of ~ 60 °C and its crystallinity and stiffness could be considerably lower compared with its bulk and the top surface of PCL80k. Consequently, the lower surface stiffness of the PCL homo-blend

could lead to lower MC3T3 cell attachment and proliferation. Surface segregation has also been found on another semi-crystalline polymer polypropylene (PP) by showing that the surface crystallinity of PP was much lower than the bulk and depth-dependent crystallinity can occur over a top layer of ~300 nm [184]. Extensive characterizations on the top surface (< 500 nm) of numerous semi-crystalline polymer systems using grazing incidence x-ray diffraction are under investigation in our research group. Meanwhile, the role of surface chemistry of the PCL homo-blends in regulating cell behavior will also be studied, in terms of hydroxyl end-groups in PCL2k.

Topological features and surface roughness at the micron and nanometer scales could influence cell behavior because the dimensions for individual cells, integrin clusters, and proteins also vary in this range [6-8]. Thermal treatment such as crystallization at different temperatures can be an effective approach to obtaining polymer substrates with controllable surface characteristics such as roughness and ordered patterns at different length scales. To demonstrate the role of surface roughness in determining cell attachment and proliferation, the PCL samples were melted and compressed between two glass plates and then cooled down below their T_m to obtain the flat disks without spherulitic structures, while rougher surfaces induced by isothermal crystallization were obtained on spin-coated thin films. By comparing the MC3T3 cell numbers at 4 h, days 1, 2, and 4 post-seeding, spherulite-roughened films of both PCL80k and PCL80k/2k ($\phi_{80k} = 75\%$) showed significantly higher cell attachment and proliferation than their

compression-smoothened counterparts because the rougher surfaces with larger surface area to allow for cells to attach and proliferate better. The increment of surface area can be reflected by higher concentrations of serum proteins adsorbed on rougher surfaces, which could further foster cell attachment and proliferation. In a previous report, silicon substrates were electrochemically etched to generate surface roughness ranging from ~2 to 100 nm, depending on the etching time [197]. Similarly, the number of attached cells and their proliferation rate both increased with roughness and approached maximum values at moderately rough surfaces.

Engineered surface nano- and micro-grooves with precise feature structures and dimensions have been used to study the “contact guidance” effect on *in vitro* cell adhesion, spreading, alignment, migration, proliferation, differentiation, and gene expression, and *in vivo* tissue development and organization [103-108]. For banded spherulites formed on the PCL samples isothermally crystallized at high temperatures, we found that this crystallization-induced concentric rings with ridges and valleys could be used to roughly arrange MC3T3 cell nucleus and the preference of nucleus location close to the valleys was dependent on the feature size, i.e. the valley depth, and cell density. However, evident cell elongation and orientation were not observed on the banded spherulites formed in this study. The possible explanation was that the valley depth was not sufficiently large and the ridge-valley structure was less uniform, compared with micro-fabricated surface patterns. Biodegradable elastomeric substrates with concentric

micro-grooves have been prepared and used for regulating MC3T3 cell behaviors and functions in our group. The results obtained from that study can be correlated with the present ones for advancing our understanding on the role of surface morphology in cell-biomaterial interactions.

5.5 Conclusions

Spin-coated thin films of PCL80k and its homo-blends with low-molecular-weight PCL2k and PCL530 have been prepared and isothermally crystallized at various temperatures. Extensive characterizations including thermal properties, surface morphologies, and crystallization kinetics, and preliminary cell studies have been performed on these samples. The melting point of PCL80k decreased progressively by blending with more PCL2k and PCL530. The crystallinity of PCL80k/2k reached a highest value when the composition of PCL2k was around 25% and 50% while the crystallinity of PCL80k/530 decreased monotonically upon blending with more amorphous component PCL530. Because of polymer lamellar twisting during crystallization, banded spherulites have been found on spin-coated thin films of pure PCL80k and its blends with PCL2k and PCL530 at proper crystallization temperatures using POM. The crystallization rate of PCL80k was increased by blending PCL2k while impeded by PCL530. The thickness of the spin-coated film was found to be strongly dependent on blend ratio because of the difference in solution viscosity. Typical

peak-valley morphology of banded spherulites with heights of ~350 and ~170 nm have been observed on the thin films of PCL80k crystallized at 52 °C and PCL80k/2k ($\phi_{80k} = 75\%$) crystallized at 50 °C, respectively. Spin-coated thin films have been found to demonstrate significantly higher surface roughness than hot-compressed samples for both PCL80k and PCL80k/2k blend ($\phi_{80k} = 75\%$). Because of the larger surface area formed in banded spherulites, protein adsorption, MC3T3 cell attachment and proliferation have been significantly enhanced. We also found that the cell nuclei tended to locate in or close to the valley of banded spherulites and this preference of localization was dependent on the feature size and cell density.

**Chapter 6. Biodegradable Elastomeric Substrates with Concentric
Microgrooves for Regulating MC3T3 Cell Behavior**

6.1 Introduction

Cell/tissue–material interactions are critical for tissue engineering applications and clinical considerations of polymeric biomaterials. There are three major categories of surface characteristics to determine cell-substrate interactions: chemical, morphological, and mechanical factors [6-8]. Surface morphologies include ordered patterns and random roughness at the micron and nanometer scales [6-8]. Many studies focused on creating substrates with nano- and micro-topographical patterns, including pits, pillars, pores, columns, and grooves, to mimic *in vivo* microenvironments and to regulate cell behavior [103-108]. These topographical features could remarkably affect *in vitro* cell adhesion, cytoskeleton organization, migration, proliferation, differentiation, and gene expression [103-108]. When cells are seeded on micro-grooved substrates, they could align, grow, or migrate along the micro-groove direction. This phenomenon is known as “contact guidance” and is dependent on cell type [109-112]. The “contact guidance” effect on osteoblast-like cells has been studied using micro-fabricated metallic and polymeric substrates to understand the interactions between bone tissue and implants and to further improve the material design strategies for fostering cellular functions and tissue organization and growth [113-118]. Because bone grafts or implants have been used to treat destructive bone diseases such as osteoporosis, osteoarthritis, and bone tumor, it is crucial to optimize the surface geometrical dimensions for promoting osteoblast cell positioning, alignment, and mineralization.

As demonstrated in Figure 6.1A, injectable, biodegradable, and photo-crosslinkable poly(ϵ -caprolactone) acrylates (PCLAs), including poly(ϵ -caprolactone) diacrylates (PCLDAs) and triacrylates (PCLTAs), have been synthesized in our research group via facile condensation between poly(ϵ -caprolactone) (PCL) diol or triol and acryloyl chloride in presence of potassium carbonate (K_2CO_3) as the proton scavenger. PCLAs prepared from PCL diols with different molecular weights can be efficiently photo-crosslinked into PCL networks, which can serve as excellent model elastomers with controllable crosslinking density, crystallinity (χ_c), and melting temperature (T_m) for regulating mechanical properties [54,83]. Compared with its amorphous counterpart, semi-crystalline PCL networks with T_m higher than 37 °C were found to be stiffer and better support mouse pre-osteoblastic MC3T3-E1 cell attachment, spreading, and proliferation [83]. Based on the enhancement effect from PCL crystallites, several other polymeric biomaterials were also prepared to achieve tunable physical properties and cell behavior for diverse tissue-engineering applications [36-39,55,57,148].

In this study, PCLTA7k and 10k synthesized from PCL triols with different molecular weights of ~7000 and ~10000 g/mol were deliberately selected to fabricate PCL networks with distinct physical properties. Crosslinked PCLTA7k is amorphous while crosslinked PCLTA10k is semi-crystalline at 37 °C. Using these two PCLTAs, we have fabricated substrates with concentric micro-grooves through replica molding from lithographed silicon wafer templates with various groove widths and depths. Mouse

pre-osteoblastic MC3T3-E1 cells have been used to examine how these two distinct substrate physicochemical characteristics and topological feature dimensions could influence cell behaviors. In the first section of this report, we present the bulk properties of crosslinked PCLTAs, including thermal, rheological, and mechanical properties. Then we demonstrate the manufacturing and morphological characterization of silicon wafers and biodegradable elastomeric crosslinked PCLTA micro-grooved substrates with various micro-groove dimensions. The rest of this report will be emphasized on MC3T3 cell attachment, spreading, proliferation, alignment, nucleus distribution, mineralization, and osteocalcin gene expression on crosslinked PCLTA substrates, in comparison with flat surfaces.

6.2 Materials and Methods

6.2.1 Synthesis and Photo-crosslinking of Poly(ϵ -caprolactone) Triacrylate (PCLTA)

All chemicals were purchased from Sigma-Aldrich (Milwaukee, WI) unless otherwise noted. PCL triol was synthesized via the ring-opening polymerization of ϵ -caprolactone at 130 °C for 12 h in the presence of $\text{Sn}(\text{Oct})_2$ as the catalyst and 1,1,1-tris(hydroxymethyl) propane (TMP) as the initiator. Different molecular weights of PCL triols can be achieved by varying the ratio of monomer and initiator. Then the PCL triol precursors were end-capped with double bonds using acryloyl chloride to obtain PCLTAs. The molecular weights of PCL triols and PCLTAs were determined by using

Gel Permeation Chromatography (GPC; PL-GPC 20, Polymer Laboratories, Inc.) at room temperature. Tetrahydrofuran (THF) was used as the eluent and standard monodisperse polystyrene samples (Polymer Laboratories) were used for calibration. PCLTA7k and 10k used in this study had number-average molecular weights (M_n) of 6700 and 9800 g/mol, weight-average molecular weights (M_w) of 8500 and 12300 g/mol, respectively.

Photo-crosslinking of PCLTA was initiated by the UV light ($\lambda = 315\text{-}380\text{ nm}$) generated from a Spectroline high-intensity long-wave UV lamp (SB-100P, Intensity: $4800\text{ }\mu\text{W}/\text{cm}^2$) in the presence of photo-initiator phenyl bis(2,4,6-trimethyl benzoyl) phosphine oxide (BAPO, Irgacure 819TM, Ciba Specialty Chemicals, Tarrytown, NY). Seventy five microliters of BAPO solution (300 mg BAPO in 1.5 mL CH_2Cl_2) was added into a pre-dissolved PCLTA/ CH_2Cl_2 solution (1.5 g/500 μL) and mixed thoroughly. PCLTA was crosslinked under UV light for 20 min at a distance of $\sim 7\text{ cm}$ from the lamp head. Crosslinked samples were soaked in acetone for two days to remove the sol fraction and completely dried in a vacuum oven.

6.2.2 Characterizations of Uncrosslinked and Photo-crosslinked PCLTAs

To determine the gel fraction of photo-crosslinked PCLTA, crosslinked PCLTA disks ($8 \times 2\text{ mm}$, diameter $\times$ thickness) were immersed in excess CH_2Cl_2 . After two days, the polymer disks were taken out and weighed after being blotted quickly. The solvent in the disks was subsequently evacuated and the dry disks were weighed. Based on the measured weights of the original (W_0) and dry (W_d) polymer disks, the gel fractions were

calculated using the equations of $W_d/W_0 \times 100\%$. Differential Scanning Calorimetric (DSC) measurements were performed on a Perkin Elmer Diamond differential scanning calorimeter in a nitrogen atmosphere to characterize the thermal properties of both uncrosslinked and photo-crosslinked PCLTAs. To keep the same thermal history, samples were first heated from room temperature to 100 °C and cooled to -90 °C at a cooling rate of 5 °C/min. Then a subsequent heating run was performed from -90 to 100 °C at a heating rate of 10 °C/min and this DSC scan was used for analysis. The tensile properties of crosslinked PCLTA specimens were implemented on a dynamic mechanical analyzer (DMTA-5, Rheometric Scientific). Briefly, polymer strips ($\sim 20 \times \sim 2 \times \sim 0.3$ mm, length $\times$ width $\times$ thickness) were elongated at a rate of 0.001 s^{-1} at 37 °C. The obtained force-displacement curves were converted to stress-strain plots and elastic moduli were calculated from the initial slopes in the plots. At least five specimens for each sample were measured and averaged.

6.2.3 Fabrication of Micro-grooved Crosslinked PCLTA Substrates

First, a reusable silicon mold was made using a standard micro-fabrication procedure, which includes photo-lithography, physical vapor deposition, lift-off, and dry plasma etching processes (Figure 6.2). Silicon was used as the substrate material for the mold. Photo-resist was applied and exposed from a pre-designed mask to define surface patterns. Then a thin aluminum layer was deposited by physical vapor deposition and a lift-off process was applied to obtain aluminum mask for silicon dry etching process. The

silicon mold was achieved by plasma dry etching and aluminum stripping. Prior to replica molding, silicon wafers were cleaned in the mixture of deionized (DI) water and ammonium hydroxide (NH_4OH) (5 parts: 1 part) at 70 °C for 10 min on a heating plate. The beaker was removed from the heating plate and another one part hydrogen peroxide (H_2O_2) was added into the mixture. The solution bubbled vigorously after 1-2 min prior to use. The silicon chip was soaked into the solution for 15 min and subsequently rinsed by DI water for 3-5 min. Finally, the silicon chip was blow-dried with nitrogen gas. PCLTA/BAPO solution was casted onto the silicon mold and then photo-crosslinked using the same procedure described in Section 6.2.1. A 2D substrate with designed pattern was fabricated by peeling off the cured polymer layer from the silicon mold.

After purification in acetone, polymer substrates were dried and flattened between two glass plates at 50 °C overnight. Finally crosslinked PCLTA substrates were sterilized in 70% ethanol solution for 6 h, dried in vacuum, and further rinsed by PBS at least three times prior to cell studies. Scanning electron microscopy (SEM; S-3500, Hitachi Instruments Inc., Tokyo, Japan) was used to characterize morphology and dimensions of micro-grooves on the crosslinked PCLTA substrates. The micro-groove dimensions were measured and averaged for at least six different locations on images for each sample.

6.2.4 Cell culture, *In Vitro* Cell Attachment, and Proliferation

Mouse MC3T3-E1 pre-osteoblastic cells (CRL-2593, ATCC, Manassas, VA) were cultured in α -Minimum Essential Media (Gibco, Grand Island, NY) supplemented with

10% FBS and 1% penicillin/streptomycin (Gibco). Cells were plated on regular tissue culture flasks with the culture media in an incubator at 37 °C with 5% CO₂ and 95% relative humidity. Cell culture media were replaced every 2-3 days and cells were split when they were 80% confluent.

MC3T3 cell attachment and proliferation on crosslinked PCLTA substrates were performed as follows. Crosslinked PCLTA samples were attached onto a petri-dish using autoclave-sterilized inert silicon-based high-temperature vacuum grease (Dow corning, Midland, MI). Cell suspension was seeded onto crosslinked PCLTA substrates at a density of 2×10^4 cells/cm² and cultured for 3 h before more culture medium was added. After the cells were cultured for 4 h, 1, 2, and 4 days, the culture medium was removed from the petri-dish and crosslinked PCLTA substrates were washed twice with PBS. Attached cells were fixed in 4% paraformaldehyde (PFA) solution for 10 min. After PFA solution was removed, the cells were washed twice with PBS and permeabilised with 0.2% (v/v) Triton X-100. Then the cells were stained with rhodamine-phalloidin (RP) for 1 h at 37 °C and 4',6-diamidino-2-phenylindole (DAPI) at room temperature for photographing on an Axiovert 25 fluorescent microscope (Carl Zeiss, Germany). Cell numbers at 4 h, days 1, 2, and 4 post-seeding were quantified by counting DAPI-stained cell nuclei on at least five fluorescent images. After culturing for two weeks, attached MC3T3 cells were fixed in methanol for 3 min, washed twice with DI water, and stained with Harris hematoxylin solution and eosin Y solution.

6.2.5 Cell Dimensions and Alignment

All the images were taken using an AxioCam ICm1 camera (Carl Zeiss, Germany) at the magnification of 100 times and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Cell length and spreading area were determined and averaged on 40 non-overlapping RP-stained cells (20 from the center and 20 from the edge of the concentric micro-grooved substrates) at 4 h post-seeding. Cells on flat substrates of crosslinked PCLTAs were used as the control groups. The length of an individual cell was determined as the longer-axis length of an ellipse that approximates the cell shape. The angle between the micro-groove direction and the direction of the longer axis of the approximated ellipse was measured over ~300 cells in different images for each sample. Cells were considered aligned if the angle was less than 15°. The percentage of cell alignment was defined as the fraction of aligned ones in the entire counted cells. The shape index or circularity of a cell nucleus, which is defined by the equation of $4 \times \pi \times \text{area}/\text{perimeter}^2$, was evaluated using the ImageJ software to quantify nuclear deformation, with a shape index of 1 representing a circle while values much less than unity correspond to high aspect ratio geometries [11]. The percentage of cell nuclei in the micro-grooves was calculated by dividing the number of cell nuclei that are majorly located in the micro-grooves by the total number of cell nuclei. At least five images were counted and averaged for analysis.

6.2.6 Cell Mineralization

After culturing for one and two weeks, MC3T3 cells were fixed in 4% PFA followed by extensive rinse with DI water to remove the non-specific precipitation. Then the cells were permeabilised with 0.2% (v/v) Triton X-100. After three times of rinse with DI water, the cells were stained with 1% (w/v) alizarin red S aqueous solution (Ricca Chemical Company, Arlington, Texas) for 10 min. Positive red staining represents calcium deposits on differentiated osteoblastic cells. Phase-contrast photographs were obtained using an Axiovert 25 light microscope (Carl Zeiss, Germany). Cell mineralization was quantified by extracting the alizarin red S stain with 100 mM cetylpyridinium chloride (MP Biomedicals, Solon, Ohio) for 2 h at room temperature. The absorbance of the extracted alizarin red S stain was measured in a 96-well plate at 570 nm on a microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA). Experiments were performed in duplicates.

6.2.7 Reverse Transcription (RT) and Real-time Polymerase Chain Reaction (PCR)

MC3T3 cells were seeded on crosslinked PCLTA substrates at a density of ~ 15000 cells/cm² and cultured for 14 days. Cells were then trypsinized and total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA). The amount of the total RNA from each sample was quantified using Nanodrop1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Reverse transcription of isolated RNA was then performed by use of DyNAmo cDNA synthesis kit (Thermo Scientific) according to the manufacturer's protocol.

Table 6.1. Oligonucleotide primer sequences for RT and real-time PCR

Gene	Primer sequence	Product Size (bp)
Osteocalcin	F: CAAGTCCCACACAGCAGCTT	371
	R: AAAGCCGAGCTGCCAGAGTT	
GAPDH	F: ACTTTGTCAAGCTCATTTC	267
	R: TGCAGCGAACTTTATTGATG	

The oligonucleotide primers used for both RT and real-time PCR were listed in Table 6.1. PCR amplification of osteocalcin and housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were conducted on a Peltier Thermo Cycler (PTC-200, MJ Research, Waltham, MA). Twenty-seven PCR cycles for osteocalcin and GAPDH were implemented with each cycle consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s. The products were analyzed by electrophoresis using 5 µL samples for 20 min at 100 V in 1.5% agarose gel stained with Gelgreen (Biotium, Hayward, CA). DNA fragments were visualized and imaged by use of EpiChemi II Darkroom Imaging Systems (UVP, Upland, CA).

Real-time PCR reactions were performed in 25 µL of PCR mixture consisting of cDNA sample, a specific primer, and a Power SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). Same thermal conditions mentioned above were applied using a Peltier Thermal Cycler with fluorescence detection systems (PTC-200, MJ Research). Expression of osteocalcin was normalized to the level of GAPDH.

6.2.8 Statistical Analysis

All data are presented as mean $\pm$ standard deviation. Student's *t*-test was performed to assess the statistical significance ($p < 0.05$) of the difference between results.

6.3. Results and Discussion

6.3.1 Characterizations of Uncrosslinked and Photo-crosslinked PCLTA

The gel fraction of a polymer network indicates the efficiency of crosslinking and decides the integrity of the sample. Without causing over-cure problems such as warping and cracks, photo-crosslinking for 20 min in the present study resulted in sufficiently high gel fractions of 0.95 ± 0.01 and 0.87 ± 0.03 for crosslinked PCLTA7k and 10k, respectively. The DSC curves of both uncrosslinked and crosslinked PCLTAs are shown in Figure 6.1C and thermal properties including T_m and heat of fusion (ΔH_m) were obtained. Among the multiple exothermal peaks in the DSC curves, the highest peak temperature was used to indicate T_m . The χ_c of crosslinked PCLTA was calculated by use of the equation $\chi_c = \frac{\Delta H_m}{\phi_{PCL} \Delta H_m^c} \times 100\%$ with ΔH_m^c of 135 J/g for completely crystalline PCL and the PCL composition (ϕ_{PCL}) of 100% [172]. Uncrosslinked PCLTA7k and 10k were semi-crystalline with T_m values of 43.4 and 50.9 °C and χ_c of 49.0% and 49.5%, respectively. Because crosslinks strongly suppressed the crystalline domains, crosslinked PCLTA7k and 10k were still semi-crystalline but with lower T_m values of 22.9 and 44.7 °C and reduced χ_c of 18.2% and 33.0%, respectively. Because of a denser network, a more significant decrease for both T_m and crystallinity was observed after crosslinking for PCLTA7k compared with PCLTA10k. Such difference made crosslinked PCLTA7k completely amorphous and transparent at physiological temperature, while crosslinked PCLTA10k was still semi-crystalline and translucent.

The distinct mechanical properties of crosslinked PCLTA strips at 37 °C are

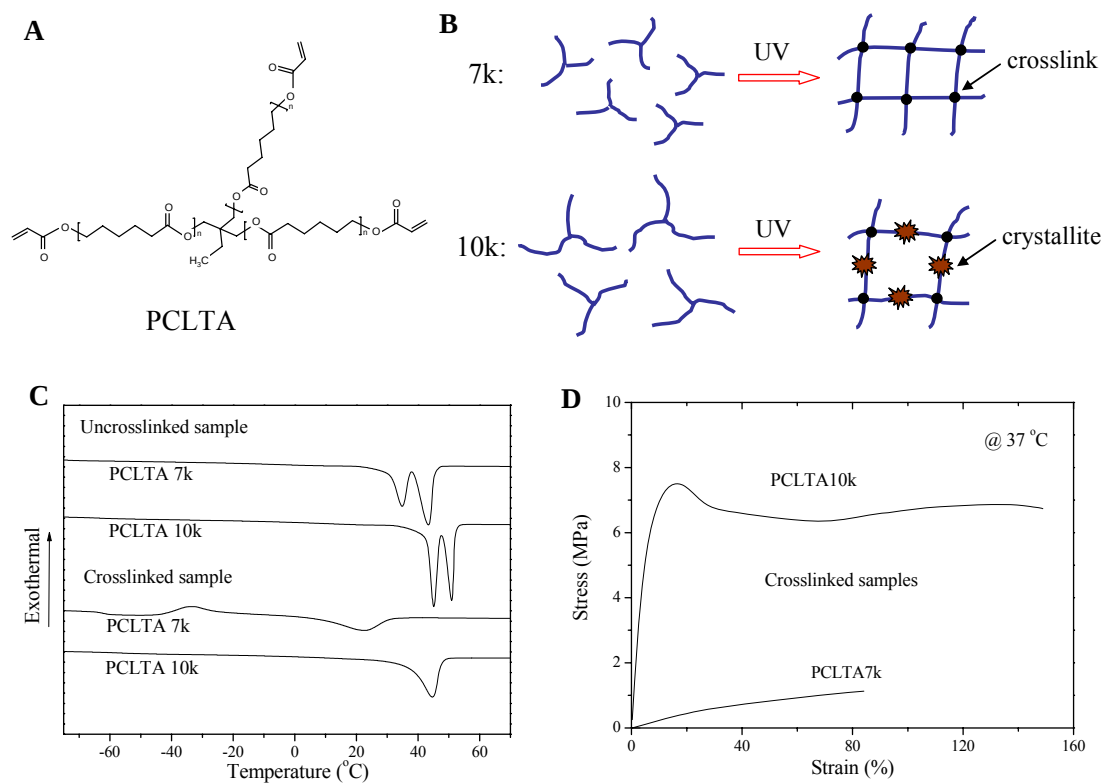


Figure 6.1. (A) Chemical structure of PCLTA. (B) Schematic difference between PCLTA7k and 10k before and after crosslinking. (C) DSC curves for uncrosslinked and crosslinked PCLTAs. (D) Representative stress-strain curves of crosslinked PCLTAs at 37 °C.

demonstrated in representative tensile stress–strain curves in Figure 6.1D. Crosslinked PCLTA7k was flexible and elastomeric at 37 °C. The tensile modulus or Young’s modulus E of crosslinked PCLTA7k was 1.7 ± 0.5 MPa, much lower than the value of 106 ± 24 MPa for crystalline crosslinked PCLTA10k with tough characteristics. Correspondingly, the stresses at break were 1.5 ± 0.3 and 9.0 ± 2.9 MPa while the strains at break were $97 \pm 13\%$ and $177 \pm 55\%$ for crosslinked PCLTA7k and 10k, respectively. These two crosslinked PCLTAs with distinct crystallinities and mechanical properties at physiological temperature can serve as excellent model polymer systems to investigate the effect of intrinsic material properties on cell behavior.

6.3.2 Crosslinked PCLTA Micro-grooved Substrates

Photo-crosslinked PCLTA substrates with micro-grooves were successfully fabricated through replica molding from micro-fabricated silicon wafers. Micro-grooved substrates with different dimensions were used in this study for investigating the effects of groove width and depth on MC3T3 cell behavior. SEM images of the micro-grooved substrates are shown in Figure 6.3-4 to determine the topographic features and dimensions. SEM images clearly showed that highly uniform concentric micro-grooves were formed over the area with a diameter of 8 mm without discernible defects and the ridges were perfectly vertical to the bottom. Deviated from the designed values of 10/10, 20/20, 50/50, and 100/100 μm , the groove width/ridge width ratios were measured to be $7.54 \pm 0.17/10.9 \pm 0.24$, $16.1 \pm 0.11/20.2 \pm 0.24$, $44.2 \pm 0.35/47.9 \pm 0.45$, and 91.2

$\pm 0.86/93.9 \pm 0.53 \text{ }\mu\text{m}$. The depths of the micro-grooves were approximately 10, 1, and $0.2 \text{ }\mu\text{m}$ as designed. Consistent with our earlier reports [39,83], crystallization-induced rougher surfaces on crosslinked PCLTA10k substrates can be observed, compared with amorphous crosslinked PCLTA7k substrates.

6.3.3 Cell Attachment and Proliferation

Fluorescent images of MC3T3 cells on the micro-grooved substrates of crosslinked PCLTA7k and 10k with the groove depth of $10 \text{ }\mu\text{m}$ as well as their flat control groups were taken, as exemplified by the cell images at 4 h post-seeding shown in Figure 6.5A. Cell numbers at 4 h, days 1, 2, and 4 on these substrates were obtained by counting nuclei in the DAPI-stained images. Similar to an earlier report [116], there was no significant difference in cell numbers at all time points between micro-grooved and flat substrates or among different micro-grooved substrates for the same crosslinked PCLTA (Figure 6.5B). Regardless of groove width, the cell numbers on crosslinked PCLTA10k substrates were always higher than those on crosslinked PCLTA7k, which might be attributed to the difference in substrate stiffness [11,12,36,39,57,83].

6.3.4 Cell Shape and Alignment

As demonstrated in Figure 6.5A, MC3T3 cells at 4 h were elongated and oriented on the micro-grooved substrates with a groove depth of $10 \text{ }\mu\text{m}$ when the groove width was 7.5 or $16.1 \text{ }\mu\text{m}$ for both crosslinked PCLTA7k and 10k. In clear contrast, cells did not show any directional preference and cytoskeletons extended radially from the nuclei on

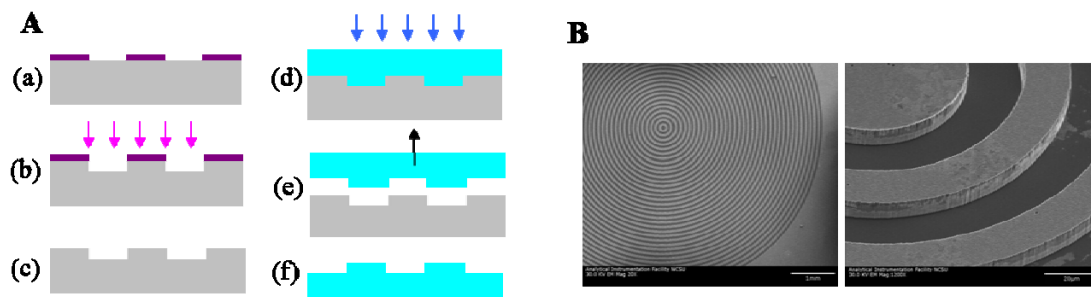


Figure 6.2. (A) Schematic micro-fabrication of silicon wafers with a pre-designed pattern and preparation of micro-grooved substrates of photo-crosslinked PCLTA via replica molding: Photolithography creates surface features on silicon substrate (a), dry etching (b), obtain silicon mold (c), pour polymer onto the mold and UV curing (d), 2D scaffold/ mold separation (e), fabricate scaffold with designed pattern (f). (B) SEM images of typical micro-fabricated silicon wafers with magnifications of $\times 20$ (left) and $\times 1200$ (right).

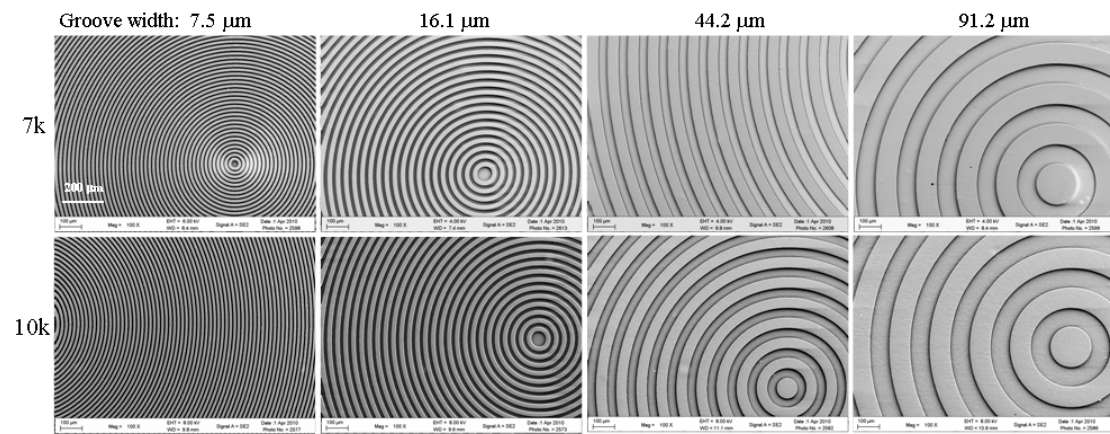


Figure 6.3. SEM images of micro-grooved PCLTA substrates with four different groove widths. Upper row: PCLTA7k; lower row: PCLTA10k. The magnification is $\times 100$ for all the images. The scale bar represents 200 μm .

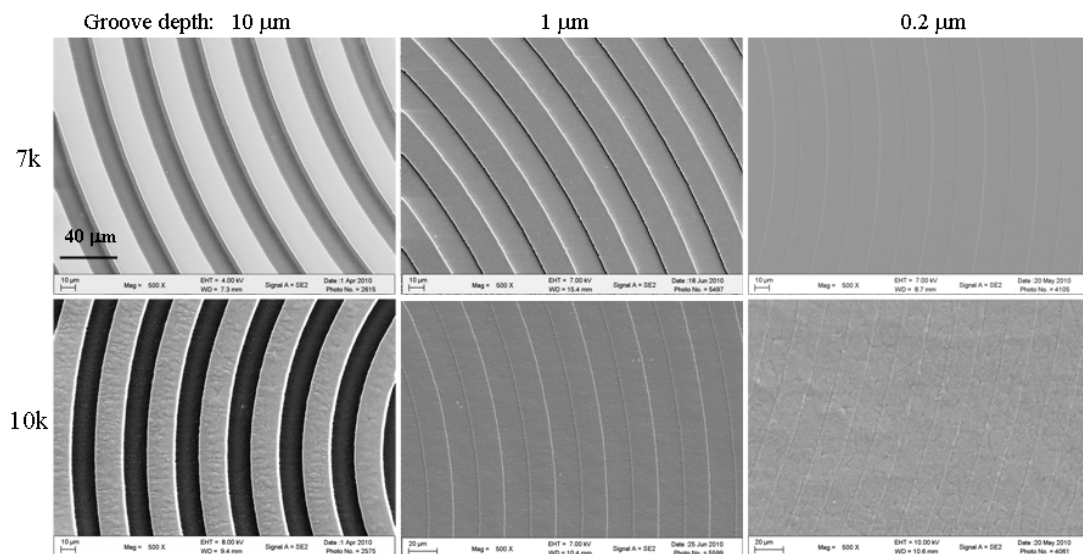


Figure 6.4. SEM images of micro-grooved PCLTA substrates (groove width: 16.1 μm) with three different groove depth. Upper row: PCLTA7k; lower row: PCLTA10k. The magnification is $\times 500$ for all the images. The scale bar represents 40 μm .

the control samples without micro-grooves. No statistical differences were found in cell length, area, and alignment between the cells located on the central and those on the edge of the micro-grooved concentric rings, although the curvature was different. When the groove width was as high as 44.2 and 91.2 μm , cell elongation and orientation could only be observed on the edge of the micro-grooves. As shown in Figure 6.5C, with large error bars, the cell length increased when the groove width decreased, on both crosslinked PCLTA7k and 10k. Cells on crosslinked PCLTA10k substrates were more spread with longer length than those on crosslinked PCLTA7k substrates with the same surface features. As shown in Figure 6.5D, cell spreading area determined at 4 h post-seeding on crosslinked PCLTA10k substrates increased with increasing the groove width but little difference can be found among various different groove widths and flat substrates of crosslinked PCLTA7k. Cells changed their preference on growth direction following the direction of the underlying micro-grooves. Figure 6.5E shows that the percentage of aligned cells on the micro-grooved substrates with groove widths of 7.5 and 16.1 μm was higher than 80% and it gradually decreased when the cells were seeded on the substrates with a larger groove width. In this study, cell elongation and alignment were more significant on narrower micro-grooves and stiffer substrates.

The alignment of MC3T3 cells was also dependent on the groove depth. Crosslinked PCLTA10k substrates with a fixed groove width of 7.5 μm and three depths of 10, 1, and 0.2 μm have been prepared (Figure 6.4) and used for examining the effect of groove

depth on cell alignment. As demonstrated in the cell images in Figure 6.7A, the percentage of aligned cells on the micro-grooved substrates decreased as the groove depth decreased from 10 to 1 μm and no obvious cell alignment could be observed when the depth was 0.2 μm .

Nucleus alignment and deformation were also observed in this study. DAPI-stained cell nuclei on the flat substrates showed no direction preference while those on the micro-grooved substrates with narrow groove widths of 7.5 and 16.1 μm were located in micro-grooves and aligned along the micro-groove direction. In contrast, micro-grooves with wider widths of 44.2 and 91.2 μm can only align the nuclei sitting close to the ridges. Similar to the cell body discussed earlier, cell nuclei were also elongated in narrow micro-grooves or along the edges of wide micro-grooves, in agreement with previous findings [198-200]. As demonstrated in Figures 6.6A and B, cells at day 4 post-seeding had smaller nuclear circularities on crosslinked PCLTA10k substrates with the groove depth of 10 μm and widths of 7.5 and 16.1 μm , compared to those on the substrates with wider micro-grooves and flat surfaces, where the cell circularity was close to 0.9. This result indicated that more significant nuclear distortion because of the confinement of narrow micro-grooves. For wider micro-grooves with widths of 44.2 and 91.2 μm , the nuclear circularity of the cells located around ridges was also significantly smaller than those on the ridges or in the micro-grooves, similar to elongation of cytoskeleton discussed earlier. A severe nuclear deformation was also found on poly(L-lactic acid)

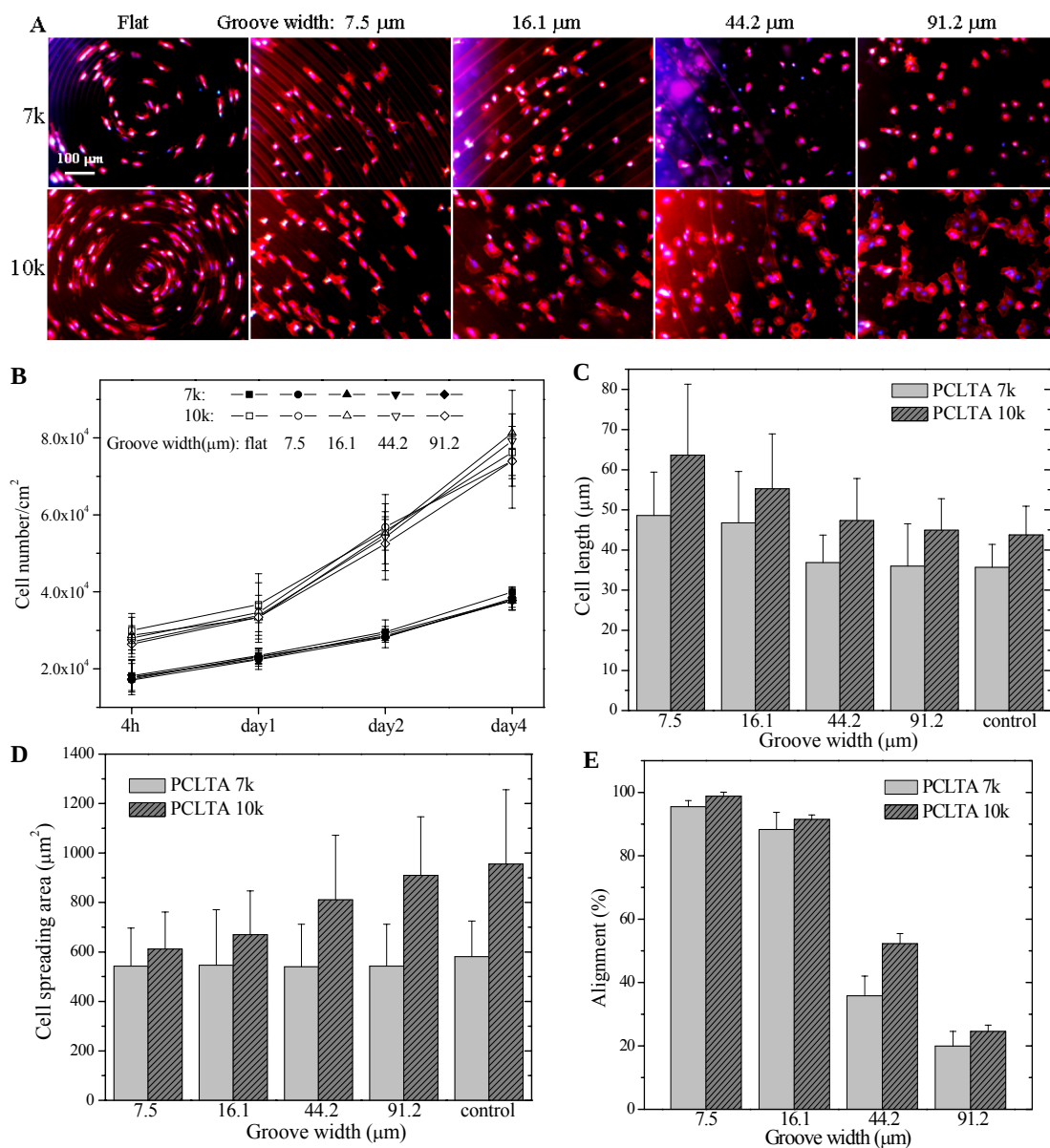


Figure 6.5. (A) Fluorescent images of MC3T3 cells stained using rhodamine-phalloidin and DAPI on flat and micro-grooved PCLTA substrates (depth: 10 μm) with four different width at 4 h post-seeding. Upper row: PCLTA7k; lower row: PCLTA10k. The scale bar represents 100 μm . (B) Number of MC3T3 cells at 4 h, days 1, 2, and 4 post-seeding. Cell length (C), spreading area (D), and alignment (E) at 4 h post-seeding.

(PLLA) substrates with pillars and it is still under investigation that whether this deformation of nucleus could result in a modification or hampering of the functioning of the organelle [201].

6.3.5 Cell Distribution

Because the ridge width was designed to be identical to the groove width, cell nuclei were observed not only in the micro-grooves but also on the ridges, especially when the groove width or the ridge width was sufficiently large. Because it was easier to locate cell nuclei than cytoplasm, cell distribution in this study was characterized using nucleus location, which can be determined by overlapping DAPI-stained cell images with phase-contrast images of the background substrates.

As shown in Figure 6.6A, for the narrowest micro-grooves with a width of 7.5 μm , only one cell nucleus spanned the width of the micro-groove and nuclei predominantly descended into the micro-grooves. In wider micro-grooves with widths of 44.2 and 91.2 μm , there were multiple cell nuclei in parallel in one micro-groove or on one ridge. For crosslinked PCLTA10k substrates with a groove depth of 10 μm , the percentage of nuclei in the micro-grooves at 4 h decreased from $95.1 \pm 1.6\%$ to $51.9 \pm 1.9\%$ when the groove width increased from 7.5 to 16.1 μm . This percentage increased gradually with the incubation time, indicating that cells preferentially migrated into the micro-grooves (Figure 6.6C). In contrast, the percentage of nuclei located in the micro-grooves of crosslinked PCLTA7k substrates was independent of culture time, suggesting the absence

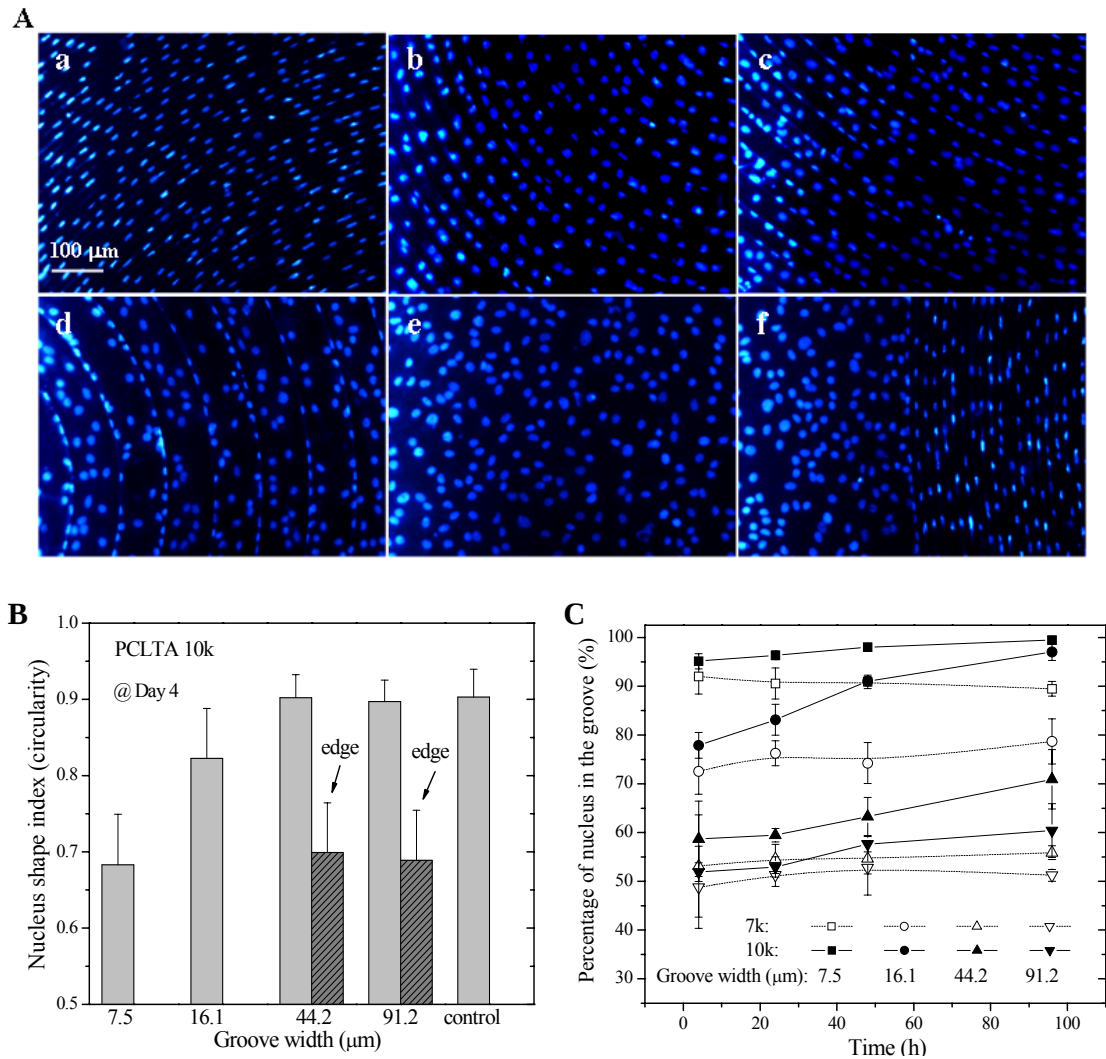


Figure 6.6. (A) Fluorescent images of cell nuclei on the micro-grooves (depth: 10 μm) with different width (a-d), flat surface (e), and intersection between flat and micro-grooved surfaces (f) of PCLTA10k at day 4 post-seeding. The scale bar represents 100 μm . (B) Shape index of nuclei on flat and micro-grooved PCLTA10k substrates at day 4 post-seeding calculated from the images in (A). (C) The percentage of cell nucleus located in the micro-grooves of PCLTAs as a function of culture time from 4 h to day 4.

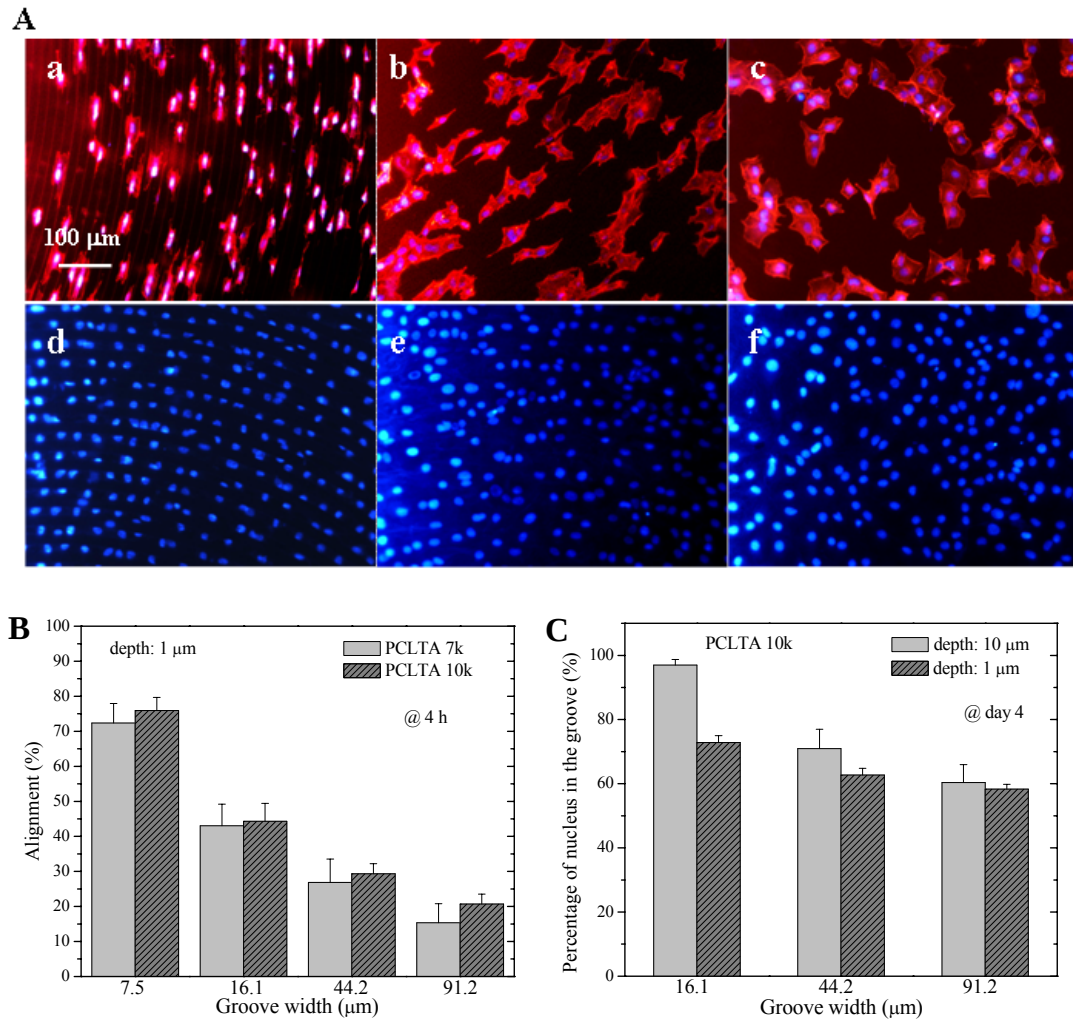


Figure 6.7. (A) Rhodamine-phalloidin stained fluorescent images on PCLTA10k micro-grooved substrates (width: 7.5 μm) with the groove depth of 10 (a), 1 (b), and 0.2 μm (c) at 4 h post-seeding and DAPI stained nuclei images on PCLTA10k micro-grooves (width: 16.1 μm) with the depth of 10 (d), 1 (e), and 0.2 μm (f) at day 4 post-seeding. The scale bar represents 100 μm. (B) Cell alignment on micro-grooved PCLTA substrates (depth: 1 μm) at 4 h post-seeding. (C) The percentage of cell nucleus located in PCLTA10k micro-grooves with the groove depth of 10 and 1 μm at day 4 post-seeding.

of the phenomenon of preferential migration (Figure 6.6C). MC3T3 cell motility was lower on more compliant substrates of crosslinked PCLTA7k while different degrees of cell migration were observed on the stiffer substrates of crosslinked PCLTA10k with various micro-grooves. The variance of percentage of nuclei in the micro-grooves was most significant when the groove width was 16.1 μm because either wider or narrower micro-grooves did not allow for such cell migration from the ridges to the micro-grooves.

Groove depth also can affect nucleus location, as demonstrated in Figure 6.7C. When the groove depth decreased from 10 to 1 μm , the percentage of nuclei completely or mostly located in the micro-grooves at day 4 decreased from $97.0 \pm 1.7\%$ to $72.8 \pm 2.2\%$, from $70.9 \pm 6.1\%$ to $62.7 \pm 2.2\%$, and from $60.4 \pm 5.5\%$ to $58.3 \pm 1.5\%$ for the groove widths of 16.1, 44.2, and 91.2 μm , respectively. When the groove depth was 1 μm and the width was 7.5 μm , the nucleus was larger than the groove width and cell nuclei cannot be confined in the micro-grooves.

6.3.6 Mineralization

Calcium content is an important indicator for quantifying mineralization of extracellular matrix (ECM) by differentiated pre-osteoblastic MC3T3-E1 cells [149,150]. In this study, the calcium deposition of MC3T3 cells on crosslinked PCLTA substrates with a groove depth of 10 μm was determined using alizarin red S solution, which is a dye to react with Ca^{2+} [202]. Alizarin red staining for mineralization was positive for all the cell-seeded substrates and the level of staining increased when the culture time was

longer because more cells differentiated and produced mineralized matrix, as exemplified by the cells cultured for 1 and 2 weeks on crosslinked PCLTA10k substrates with a groove width of 16.1 μm (Figure 6.8).

The cells seeded on the same substrates and cultured for 2 weeks were also stained with Harris hematoxylin solution and eosin Y solution to demonstrate the morphologies of aligned cells in the micro-grooves and non-aligned cells on the flat margin (Figure 6.8Ac). Interestingly, with a similar cell density, the mineralization in the micro-grooves was significantly higher than that on the flat margin. In addition, a higher staining intensity on crosslinked PCLTA10k substrates was found in comparison to crosslinked PCLTA7k (Figure 6.8Ab and d), in agreement with the results that pre-osteoblastic cell differentiation favored stiffer substrates.

Groove width played a critical role in influencing MC3T3 cell differentiation. To demonstrate this effect clearly, images were taken from the intersection to include both micro-grooves and the flat margin of crosslinked PCLTA10k substrates with a groove depth of 10 μm and different widths after cells were cultured for 2 weeks. As shown in Figures 6.8Ae-h, MC3T3 cells produced the densest mineral nodules in narrow micro-grooves with widths of 7.5 and 16.1 μm . The difference in the staining intensity between micro-grooves and flat margins became smaller when the groove width increased. Quantitatively demonstrated in Figure 6.8B, the alizarin red stain extracted from stiffer substrates or the narrower micro-grooves had significant higher absorbance

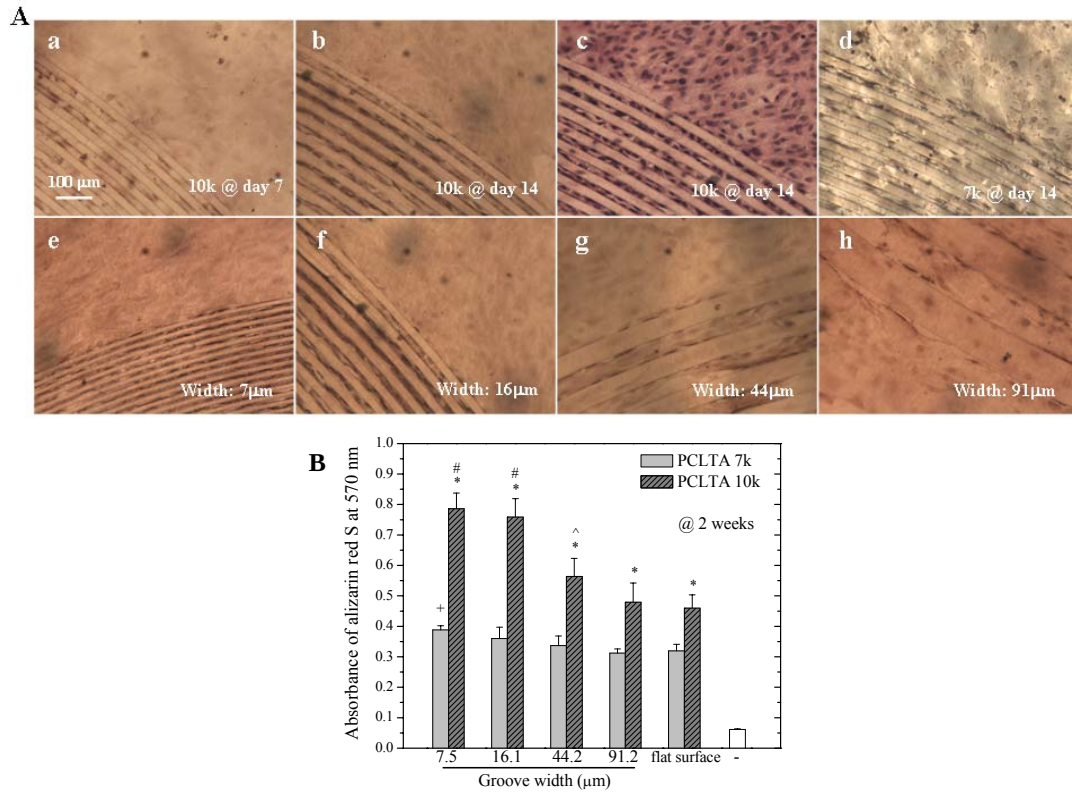


Figure 6.8. (A) Alizarin red S stained images of PCLTA10k (width: 16.1 μm) at 1 week (a), 2 weeks (b), and PCLTA7k (width: 16.1 μm) at 2 weeks (d); Harris hematoxylin solution and eosin Y solution stained image of PCLTA10k (width: 16.1 μm) at 2 weeks (c); images taken on the intersection between flat and micro-grooved surfaces with the groove width of 7.5 (e), 16.1 (f), 44.2 (g), and 91.2 μm (h) at 2 weeks. (B) Quantification of Alizarin red S extracted from flat and micro-grooved PCLTAs with the depth of 10 μm at 2 weeks. Negative control is the pure cetylpyridinium chloride solution. * $p < 0.05$, relative to the counterpart of PCLTA7k, # $p < 0.05$ relative to flat and micro-grooved PCLTA10k with the width of 44.2 and 91.2 μm , ^ $p < 0.05$ relative to flat PCLTA10k, + $p < 0.05$ relative to micro-grooved PCLTA7k with the width of 91.2 μm .

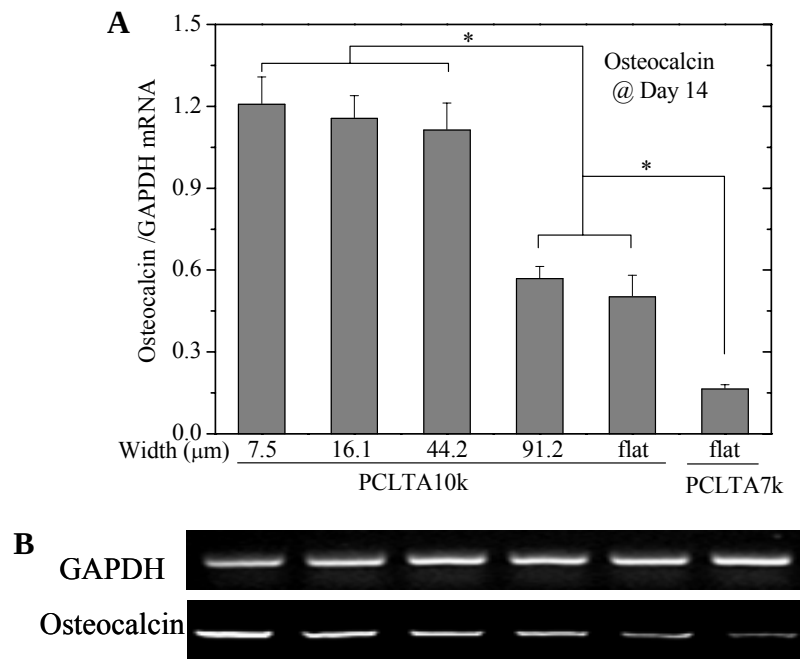


Figure 6.9. Osteocalcin gene expression normalized by GAPDH in MC3T3 cells cultured on flat and micro-grooves of PCLTA10k and flat PCLTA7k substrate for 14 days by real time (A) and RT PCR (B) analysis.

values.

6.3.7 Osteocalcin Gene Expression

As demonstrated in Figures 6.9A and B, the expression of osteocalcin gene was evaluated by both real-time and RT PCR using total RNA extracted from MC3T3 cells cultured on crosslinked PCLTA substrates for 14 days. It is evident that the expression level of osteocalcin gene was significantly higher for MC3T3 cells cultured on micro-grooved substrates with smaller groove widths. When the groove width was 91.2 μm , there was even no significant difference from that on the flat control group. As expected, stiffer substrates of semi-crystalline crosslinked PCLTA10k always had higher gene expression levels of osteocalcin compared with the substrates made from amorphous crosslinked PCLTA7k.

6.4 Further Discussion

Photo-crosslinking PCLTA on micro-fabricated silicon wafers with pre-designed patterns was efficient for producing defect-free micro-grooved biodegradable polymer substrates with highly uniform structures and precise dimensions. Crosslinked PCLTA substrates with micro-grooves can be easily peeled off from the silicon wafers. These micro-grooved substrates with concentric rings can be used to mimic similar self-assembled patterns found in polymers, peptides, and inorganic crystals. For example, banded spherulites with ridges and valleys can be formed when PCL crystallizes slowly

at a temperature slightly lower than its T_m . The purpose of this study was also to explore how pre-osteoblastic cells respond to both different substrates and micro-fabricated features. No significant difference was found in cell alignment between the center part and the edge part of the concentric rings.

For crosslinked PCLTA10k, PCL crystalline domains formed a physical network to dramatically strengthen the chemical network at physiological temperature. In contrast, crosslinked PCLTA7k only contained a chemical network without being enhanced by crystallites at physiological temperature because its T_m was lower than 37 °C. As elaborated in our earlier reports [39,83], a higher surface stiffness might be the reason for better MC3T3 cell attachment, spreading, and proliferation on semi-crystalline crosslinked PCLTA10k substrates compared with their amorphous PCLTA7k counterparts. By controlling the molecular weight of PCLTA, we can achieve crosslinked PCLTAs with a wide range of mechanical properties and use them to regulate cell behavior and satisfy the requirements from soft tissue replacement to hard tissue replacement.

By adjusting their phenotype to the substrate texture, cells can be aligned in micro-grooves with suitable dimensions and such alignment varies when the cell type is different [110-112]. Pre-osteoblastic cell behavior on polymer substrates with ordered groove/ridge features have been studied extensively [113-118]. Cell alignment and migration along the micro-groove direction could be observed when both groove depth and width were at the micro- or nanoscopic level. Our findings that MC3T3 cells were

responsive to the micro-grooves were consistent with literature reports. If the groove spacing is wide ($> 5 \mu\text{m}$) but the depth is less than $1 \mu\text{m}$, cell skeleton may not span enough ridges and grooves or sense the surface roughness and topography. It may be the reason for that MC3T3 cells did not respond to the micro-grooves with a depth of $0.2 \mu\text{m}$ in this study. Osteoblast migration is crucial in endochondral or intramembranous bone formation, bone remodeling, and repair of bone tissue [203]. The percentage of cell nuclei located in the micro-grooves of crosslinked PCLTA10k increased with culturing time while it did not change on crosslinked PCLTA7k substrates, indicating that MC3T3 cells were liable to migrate to the micro-grooves after cell adhesion and spreading. Such migration strongly depended on the material properties.

The differentiation of osteoblasts was not only influenced by the surface mechanics but also by the presence of micro-grooves. Stiffer crosslinked PCLTA10k substrates in this study showed significantly higher MC3T3 cell differentiation as well as cell attachment and proliferation than crosslinked PCLTA7k. It has been reported that the alkaline phosphatase (ALP) activity and mineralization of osteoblasts on numerous materials can be enhanced by surface features [114,119-122]. Rough-textured and porous-coated titanium surfaces can enhance both synthesis and mineralization of the ECM *in vitro* [119]. Assessed by radiography and electron microscopy at the implant interface, *in vivo* mineralization occurred often on grooved, pitted, and polished titanium surfaces but rarely on smooth ones when they were implanted [120,121]. Calcium

deposits from rat bone marrow cells (rBMCs) can be clearly seen inside the denser groove spacing compared with smooth polystyrene substrates from transmission electron microscopic images [114]. The ALP activity of rBMCs showed higher mineralized ECM deposit on grooved surfaces of PLLA with a depth of 1 μm and a width of 1 or 2 μm than non-grooved substrates [122]. In this study, micro-grooved crosslinked PCLTA substrates substantially enhanced the mineralization process of MC3T3 cells compared to flat substrates. Narrow micro-grooves with widths of 7.5 and 16.1 μm and a depth of 10 μm showed significantly higher differentiation results on both crosslinked PCLTA7k and 10k. The mineral deposition by osteoblasts in an aligned fashion can also be found on nano-size grooves reported previously [204].

Cellular functions can be altered by regulating cell phenotype. More elongated osteoblast-like and fibroblast cells on micro-grooved substrates could express a higher level of collagen type I, one important protein in ECM produced by cells, than less or non-stretched cells [117,205,206]. The collagenous matrix could also be aligned along the micro-groove direction through controlling actin cytoskeletal structure, focal adhesion distribution, and traction forces in cells [117,205,206]. The aligned osteoblast cells also could promote the development of functional cell polarity which favors mineralization and subsequent bone formation [121]. In our present study, highly oriented cells in narrower micro-grooves with widths of 7.5 and 16.1 μm showed higher differentiation results and higher expression levels of osteocalcin gene compared with wider

micro-grooves. Because more MC3T3 cells were trapped in the narrower micro-grooves, cell-cell communications were better and regulatory factors could reach concentrations that promote calcified tissue formation [121]. It has been reported that paired or aggregated bone marrow stromal cells exhibited more significant differentiation than isolated ones and the extent of differentiation was almost linearly related to the extent of contact [207]. In addition, alignment can up-regulate specific gene expression for oriented cells on nano- or micro-sized grooves [208-210].

6.5 Conclusions

Photo-crosslinkable PCLTAs have been used as an excellent model system to achieve controllable physicochemical characteristics for elucidating the relationship between intrinsic material properties and cell behaviors. Concentric micro-grooved substrates of photo-crosslinked PCLTAs with different groove widths and depths have been fabricated through replica molding from silicon wafers. Micro-grooved crosslinked PCLTA substrates with precise surface patterns have served effectively in investigating the topographical cue in regulating osteoblast-like cell alignment, distribution, and directional mineralization for bone tissue engineering applications. At 37 °C, crosslinked PCLTA10k network was semi-crystalline and stiff while crosslinked PCLTA7k was amorphous and compliant. Crosslinked PCLTA10k substrates could better support rat pre-osteoblastic MC3T3-E1 cell attachment, proliferation, differentiation than the

substrates of crosslinked PCLTA7k, regardless of micro-groove dimensions. Cell contact guidance, cytoskeletal architecture, nucleus shape and location, mineralization, and gene expression level of osteocalcin have been found to be more significant on the narrower micro-grooves with a width of 7.5 μm and a depth of 10 μm . These results indicate that both intrinsic material properties and surface micro-grooves have significant impacts on regulation of MC3T3 cell behaviors. The present results further offer guidance for fabricating 3D scaffolds for promoting bone repair and regeneration.

Chapter 7. Conclusion

Novel injectable and photo-crosslinkable hybrid organic-inorganic composites of PPF and mPOSS were prepared for bone tissue engineering applications. Liquid-like mPOSS can behavior as a crosslinker to enhance the photo-crosslinking efficiency of PPF due to its eight crosslinkable end groups on one nano-cage, decrease the zero-shear viscosity of uncrosslinked PPF, regulate mechanical properties and accelerate the hydrolytic degradation of crosslinked PPF/mPOSS networks. Blending mPOSS did not significantly alter surface wettability and serum protein adsorption. Similar to crosslinked PPF, crosslinked PPF/mPOSS could support MC3T3 cell attachment, spreading, proliferation, and differentiation and showed no significant difference among the samples with different mPOSS compositions. Interestingly, crosslinked HPPF/mPOSS showed distinct physical properties and promoted MC3T3 cell functions compared with their LPPF counterparts because of the higher crosslinking densities in networks.

Instead of PPF/mPOSS networks with only chemical crosslinks existing, semi-interpenetrating polymer networks were formed by introducing semicrystalline PCL into amorphous PPF which combined both PPF chemical network and PCL physical network that was associated by the crystallites. PPF softened PCL in the uncrosslinked blends but it strengthened PCL after cross-linking, resulting in a wide range of mechanical properties of PPF/PCL blends. Because of the PCL crystallization, surface morphology can be significantly roughened by increasing the composition of PCL. Distinct cell responses were found to the PPF/PCL blends in the presence or absence of

crosslinks. At the same composition, crosslinked PPF/PCL blends could support MC3T3 cell attachment, spreading, and proliferation significantly better than their uncrosslinked counterparts due to the effect of stiffness. In uncrosslinked blends, cell attachment and proliferation were dramatically reduced by blending PPF with PCL and such a decrease was much more dramatic compared to the composition dependence of the bulk tensile modulus, suggesting that surface segregation occurred and resulted in a top layer rich in more mobile PPF and a lower surface stiffness compared to the bulk.

Banded spherulites showing concentric periodic bands along the radial direction due to polymer lamellae twisting were found on spin-coated thin films of PCL80k and its blends with PCL2k, PCL530, and amorphous PPF at appropriate crystallization temperature. Thermal properties, crystallization rate, and banded spherulitic morphology of these blends can be well regulated by using isothermal crystallization temperature and blend composition. Pure PCL80k, PCL80k/2k ($\phi_{80k} = 75\%$), and PCL80k/PPF ($\phi_{80k} = 75\%$) were selected to investigate the effect of banded spherulites on MC3T3 cell behaviors. The roughness of the crystals formed on the surface of these samples increased with T_c , resulting in enhanced protein adsorption from cell culture medium due to the increased surface area induced by banded spherulites. MC3T3 cell attachment and proliferation did not show significant difference among different PCL80k/PPF ($\phi_{PCL80k} = 75\%$) samples isothermally crystallized at 25, 37, and 45 °C with R_{rms} ranging from 27.4 to 37.1 nm. In order to investigate the effect of roughness on cell responses in a wider

range, hot-compressed disks with relatively flat surface were prepared for both PCL80k and PCL80k/2k blend. R_{rms} values were 17.6, 36.0, and 72.9 nm for hot-compressed PCL80k disk and the films crystallized at 25 and 52 °C, while they were 20.6, 32.8, and 48.1 nm for hot-compressed PCL80k/2k ($\phi_{80k} = 75\%$) disk and the films crystallized at 25 and 50 °C, respectively. For these samples, spherulite-roughened films could support cell attachment and proliferation significantly better than compression-smoothened disks due to the larger difference of surface roughness. Although crystallization-induced peak-valley morphology was clearly found, cell contact guidance phenomenon was not observed on the banded spherulites formed in these hetero- and homo-blends containing PCL. Similar to uncrosslinked PPF/PCL blends, surface segregation could occur in PCL80k/2k blend ($\phi_{80k} = 75\%$) resulting in significantly decreased cell attachment and proliferation compared to pure PCL80k.

In order to simulate the typical peak-valley structure of banded spherulites and subsequently regulate cellular responses, micro-structured substrates of photo-crosslinked PCLTAs with concentric ridge-groove patterns were fabricated through replica molding from silicon wafers. Photo-crosslinked PCLTA7k and 10k with the melting temperature lower and higher than 37 °C resulted in their distinct crystallinities and mechanical properties at physiological temperature. Regardless of micro-groove dimensions, semi-crystalline and stiff PCLTA10k substrates could better support MC3T3 cell attachment, proliferation, differentiation than amorphous and compliant PCLTA7k

substrates which was consistent with the stiffness effect on cell responses demonstrated in PPF/mPOSS and PPF/PCL blends. Because of the more uniform structure and larger feature dimensions, micro-grooves on PCLTAs could regulate cell behaviors more effectively than banded spherulites obtained in PCL hetero- and homo-blends. In addition, cell contact guidance, cytoskeletal architecture, nucleus shape and location, mineralization, and gene expression level of osteocalcin were more significant on the narrower micro-grooves with widths of 7.5 and 16.1 μm and a depth of 10 μm . These results could offer important guidance for fabricating 3D scaffolds for promoting bone repair and regeneration.

Polymer crosslinking density and crystallinity are critical in determining surface characteristics such as hydrophilicity, protein adsorption, stiffness, and morphology. These controllable surface characteristic, serving as important factors to influence cell/biomaterial interactions, can be used to consequently regulate cell behaviors and functions. Considering many polymeric biomaterials are semi-crystalline, this research aspect should be emphasized in the interdisciplinary field of tissue engineering.

References

1. Langer, R.; Vacanti, J. P. *Science* **1993**, *260*, 920.
2. Kneser, U.; Schaefer, D. J.; Munder, B.; Klemmt, C.; Andree, C.; Stark, G. B. *Min Invas Ther Allied Technol* **2002**, *11*, 107.
3. Puppi, D.; Chiellini, F.; Piras A. M.; Chiellini, E. *Progress in Polymer Science* **2010**, *35*, 403.
4. Sabir, M. I.; Xu, X.; Li, L. *Journal of Materials Science* **2009**, *44*, 5713.
5. Griffith, L. G. *Acta Materialia* **2000**, *48*, 263.
6. Harbers, G. M.; Grainger, D. W. "Cell-Material Interactions: Fundamental Design Issues for Tissue Engineering and Clinical Considerations" In *Introduction to Biomaterials* Guelcher, S. A.; Hollinger, J. O. Eds. Boca Raton: CRC Press, 2005, pp. 15-45.
7. Wong, J. Y.; Leach, J. B.; Brown, X. Q. *Surf. Sci.* **2004**, *570*, 119.
8. Saltzman, W. M., Kyriakides, T. R. Cell interactions with polymers. In: Lanza, R.; Langer, R.; Vacanti, J. Eds. *Principles of tissue engineering*. 3rd ed., San Diego: Elsevier Academic Press, 2007. p. 279-96.
9. Lee, S. J.; Khang, G.; Lee, Y. M.; Lee, H. B. *Biomaterials* **2003**, *259*, 228.
10. Brocchini, S.; James, K.; Tangpasuthadol, V.; Kohn, J. *J Biomed Mater Res* **1998**, *42*, 66.
11. Discher, D. E.; Janmey, P.; Wang, Y.-L. *Science* **2005**, *310*, 1139.
12. Pelham, R. J. Jr.; Wang Y.-L. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 13661.

13. Pelham, R. J. Jr.; Wang Y.-L. *Biol. Bull.* **1998**, *194*, 348.
14. Peter, S. J.; Miller, M. J.; Yasko, A. W.; Yaszemski, M. J.; Mikos, A. G. *J Biomed Mater Res* **1998**, *43*, 422.
15. Khan, Y.; Yaszemski, M. J.; Mikos, A. G.; Laurencin, C. T. *J Bone Joint Surg Am* **2008**, *90*, 36.
16. Mousa, W. F.; Kobayashi, M.; Shinzato, S.; Kamimura, M.; Neo, M.; Yoshihara, S.; Nakamura. *Biomaterials* **21**, 2137, 2000.
17. Ifkovits, J. L.; Burdick, J. A. *Tissue Engineering* **2007**, *13*, 2369.
18. Anseth, K. S.; Shastri, V. R.; R. Langer. *Nature Biotechnology* **1999**, *17*, 156.
19. Fisher, J. P.; Dean, D.; Engel, P. S.; Mikos, A. G. *Ann Rev Mater Res* **2001**, *31*, 171.
20. Amsden, B. *Soft Matter* **2007**, *3*, 1335.
21. Shi, X. F.; Mikos, A. G. "Poly(propylene fumarate)" In *Introduction to Biomaterials*. Guelcher, S. A.; Hollinger, J. O.; Eds; Boca Raton: CRC Press, 2006, pp. 205-218.
22. Shung, A. K.; Timmer, M. D.; Jo, S.; Engel, P. S.; Mikos, A. G. *J Biomater Sci Polymer Edn* **2002**, *13*, 95.
23. Kasper, F. K.; Tanahashi, K.; Fisher, J. P.; Mikos, A. G. *Nat Protoc* **2009**, *4*, 518.
24. Wang, S.; Lu, L.; Yaszemski, M. J. *Biomacromolecules* **2006**, *7*, 1976.
25. He, S. L.; Timmer, M. D.; Yaszemski, M. J.; Yasko, A. W.; Engel, P. S.; Mikos, A. G. *Polymer* **2001**, *42*, 1251.
26. Timmer, M. D.; Ambrose, C. G.; Mikos, A. G. *Biomaterials* **2003**, *24*, 571.

27. Lee, K.-W.; Wang, S.; Lu, L.; Jabbari, E.; Currier, B. L.; Yaszemski, M. J. *Tissue Eng* **2006**, *12*, 2801.
28. Lee, K.-W.; Wang, S.; Yaszemski, M. J.; Lu, L. *Biomacromolecules* **2010**, *11*, 682.
29. Lee, K.-W.; Wang, S.; Fox, B. C.; Ritman, E. L.; Yaszemski, M. J.; Lu, L. *Biomacromolecules* **2007**, *8*, 1077.
30. Cook, M. N.; Fisher, J. P.; Dean, D.; Rimnac, C.; Mikos, A. G. *J Biomed Mater Res Part B Appl Biomater* **2003**, *64*, 65.
31. Lee, J. W.; Lan, P. X.; Kim, B.; Lim, G.; Cho, D. W. *Microelectron Eng* **2007**, *84*, 1702.
32. Lee, J. W.; Lan, P. X.; Kim, B.; Lim, G.; Cho, D. W. *J Biomed Mater Res Part B: Appl Biomater* **2008**, *87B*, 1.
33. Lee, J. W.; Kang, K. S.; Lee, S. H.; Kim, J.-Y.; Lee, B.-K.; Cho, D.-W. *Biomaterials* **2011**, *32*, 744.
34. He, S. L.; Yaszemski, M. J.; Yasko, A. W.; Engel, P. S.; Mikos, A. G. *Biomaterials* **2000**, *21*, 2389.
35. Fisher, J. P.; Dean, D.; Mikos, A. G. *Biomaterials* **2002**, *23*, 4333.
36. Wang, S.; Kempen, D. H.; Simha, N. K.; Lewis, J. L.; Windebank, A. J.; Yaszemski, M. J.; Lu, L. *Biomacromolecules* **2008**, *9*, 1229.
37. Wang, S.; Lu, L.; Gruetzmacher, J. A.; Currier, B. L.; Yaszemski, M. J. *Macromolecules* **2005**, *38*, 7358.

38. Cai, L.; Wang, K.; Wang, S. *Biomaterials* **2010**, *31*, 4457.
39. Cai, L.; Wang, S. *Biomaterials* **2010**, *31*, 7423.
40. Lee, K. W.; Wang, S.; Yaszemski, M. J.; Lu, L. *Biomaterials* **2008**, *29*, 2839.
41. Hakimimehr, D.; Liu, D. M.; Troczynski, T. *Biomaterials* **2005**, *26*, 7297.
42. Cai, Z.; Yang, D. A.; Zhang, N.; Ji, C. G.; Zhu, L.; Zhang, T. *Acta Biomater* **2009**, *5*, 628.
43. Cai, Z.; Zhang, T.; Di, L.; Xu, D. M.; Xu, D. H.; Yang, D. A. *Biomed Microdevices* DOI 10.1007/s10544-011-9532-8.
44. Chang, C.-H.; Liao, T.-C.; Hsu, Y.-M.; Fang, H.-W.; Chen, C.-C.; Lin, F.-H. *Biomaterials* **2010**, *31*, 4048.
45. Peter, S. J.; Lu, L.; Kim, D. J.; Mikos, A. G. *Biomaterials* **2000**, *21*, 1207.
46. Shi, X.; Sitharaman, B.; Pham, Q. P.; Liang, F.; Wu, K.; Billups, W. E.; Wilson, L. J.; Mikos, A. G. *Biomaterials* **2007**, *28*, 4078.
47. Cruise, G. M.; Scharp, D. S.; Hubbell, J. A. *Biomaterials* **1998**, *19*, 1287.
48. Jo, S.; Shin, H.; Shung, A. K.; Fisher, J. P.; Mikos, A. G. *Macromolecules* **2001**, *34*, 2839.
49. Wang, S.; Lu, L.; Gruetzmacher, J. A.; Currier, B. L.; Yaszemski, M. J. *Biomaterials* **2006**, *27*, 832.
50. Bohl, K. S.; West, J. L. *Biomaterials* **2000**, *21*, 2273.
51. Carnahan, M. A.; Middleton, C.; Kim, J.; Kim, T.; Grinstaff, M. W. *J Am Chem Soc*

2002, 124, 5291.

52. Degoricija, L.; Johnson, C. S.; Wathier, M.; Kim, T.; Grinstaff, M. W. *Investigative Ophthalmology & Visual Science* **2007**, 48, 2037.
53. Degoricija, L.; Bansal, P. N.; Sontjens, S. H. M.; Joshi, N. S.; Takahashi, M.; Snyder, B.; Grinstaff, M. W. *Biomacromolecules* **2008**, 9, 2863.
54. Cai, L.; Wang, S. *Biomacromolecules* **2010**, 11, 304.
55. Wang, S.; Yaszemski, M. J.; Gruetzmacher, J. A.; Lu, L. *Polymer* **2008**, 49, 5692.
56. Wang, S.; Kempen, D. H.; Yaszemski, M. J.; Lu, L. *Biomaterials* **2009**, 30, 3359.
57. Wang, S.; Yaszemski, M. J.; Knight, A. M.; Gruetzmacher, J. A.; Windebank, A. J.; Lu, L. *Acta Biomaterialia* **2009**, 5, 1531.
58. Metter, R. B.; Ifkovits, J. L.; Hou, K.; Vincent, L.; Hsu, B.; Wang, L.; Mauck, R. L.; Burdick, J. A. *Acta Biomaterialia* **2010**, 6, 1219.
59. Lynn, D. M.; Langer, R. *J Am Chem Soc* **2000**, 122, 10761.
60. Lynn, D. M.; Anderson, D. G.; Putnam, D.; Langer, R. *J Am Chem Soc* **2001**, 123, 8155.
61. Anderson, D. G.; Tweedie, C. A.; Hossain, N.; Navarro, S. M.; Brey, D. M.; Van Vliet, K. J.; Langer, R.; Burdick, J. A. *Advanced Materials* **2006**, 18, 2614.
62. Brey, D. M.; Erickson, I.; Burdick, J. A. *Journal of Biomedical Materials Research: Part A* **2008**, 85A, 731.
63. Muhlebach, A.; Muller, B.; Pharisa, C.; Hofmann, M.; Seiferling, B.; Guerry, D.

Journal of Polymer Science Part A: Polymer Chemistry **1997**, *35*, 3603.

64. Bryant, S. J.; Davis-Arehart, K. A.; Luo, N.; Shoemaker, R. K.; Arthur, J. A.; Anseth, K. S. *Macromolecules* **2004**, *37*, 6726.
65. Schmedlen, R. H.; Masters, K. S.; West, J. L. *Biomaterials* **2002**, *23*, 4325.
66. Martens, P. J.; Bryant, S. J.; Anseth, K. S. *Biomacromolecules* **2005**, *4*, 283.
67. Anseth, K. S.; Metters, A. T.; Bryant, S. J.; Martens, P. J.; Elisseeff, J. H.; Bowman, C. N. *Journal of Controlled Release* **2002**, *78*, 199.
68. Nuttelman, C. R.; Henry, S. M.; Anseth, K. S. *Biomaterials* **2002**, *23*, 3617.
69. Muggli, D. S.; Burkoth, A. K.; Keyser, S. A.; Lee, H. R.; Anseth, K. S. *Macromolecules* **1998**, *31*, 4120.
70. Anseth, K. S.; Shastri, V. R.; Langer, R. *Natural Biotechnology* **1999**, *17*, 156.
71. Young, J. S.; Gonzales, K. D.; Anseth, K. S. *Biomaterials* **2000**, *21*, 1181.
72. Burkoth, A. K.; Burdick, J.; Anseth, K. S. *Journal of Biomedical Materials Research* **2000**, *51*, 352.
73. Poshusta, A. K.; Burdick, J. A.; Mortisen, D. J.; Padera, R. F.; Ruehlman, D.; Yaszemski, M. J.; Anseth, K. S. *Journal of Biomedical Materials Research* **2003**, *64A*, 62.
74. Burkoth, A. K.; Anseth, K. S. *Biomaterials* **2000**, *21*, 2395.
75. Engelberg, I.; Kohn, J. *Biomaterials* **1991**, *12*, 292.
76. Perrin, D. E.; English, J. P. "Polycaprolactone" In *Handbook of Biodegradable*

Polymers; Domb, A. J.; Kost, J., Wiseman, D.; Eds; Amsterdam: Harwood Academic Publishers, 1997, pp. 63-77.

77. Wang, K.; Cai L.; Wang, S. *Abstr. Pap. Am. Chem. Soc.* **2010**, *240*, PMSE-345.
78. Wang, K.; Cai L.; Wang, S. *Abstr. Pap. Am. Chem. Soc.* **2010**, *103*, PMSE-536.
79. Ciardelli, G.; Chiono, V.; Vozzi, G.; Pracella, M.; Ahluwalia, A.; Barbani, N.; Cristallini, C.; Giusti, P. *Biomacromolecules* **2005**, *6*, 1961.
80. Tang, Z. G.; Hunt, J. A. *Biomaterials* **2006**, *27*, 4409.
81. Calvert, J. W.; Marra, K. G.; Cook, L.; Kumta, P. N.; DiMilla, P. A.; Weiss, L. E. *J. Biomed. Mater. Res.* **2000**, *52*, 279.
82. Shao, H.-J.; Lee, Y.-T.; Chen, C.-S.; Wang, J.-H.; Young, T.-H. *Biomaterials* **2010**, *31*, 4695.
83. Cai, L.; Wang S. *Polymer* **2010**, *51*, 164.
84. Keller, A. *J. Polym. Sci.* **1955**, *17*, 291.
85. Keith, H. D.; Padden, F. J. Jr. *Polymer* **1984**, *25*, 28.
86. Keith, H. D. *Polymer* **2001**, *42*, 9987.
87. Lotz, B.; Cheng, S. Z. D. *Polymer* **2005**, *46*, 577.
88. Woo, E. M.; Mandal, T. K.; Lee, S. C. *Colloid Polym. Sci.* **2000**, *278*, 1032.
89. Su, C. C.; Lin, J. H. *Colloid Polym. Sci.* **2004**, *283*, 182.
90. Wang, Z.; Alfonso, G. C.; Hu, Z.; Zhang, J.; He, T. *Macromolecules* **2008**, *41*, 7584.
91. Keith, H. D.; Padden, F. J. Jr.; Russell, T. P. *Macromolecules* **1989**, *22*, 666.

92. Neo, M. K.; Goh, S. H. *Eur. Polym. J.* **1991**, *27*, 927.
93. Wang, Z.; Jiang, B. *Macromolecules* **1997**, *30*, 6223.
94. Cheung, Z. L.; Weng, L. T.; Chan, C. M.; Hou, W. M.; Li, L. *Langmuir* **2005**, *21*, 7968.
95. Yam, W. Y.; Ismail, J.; Schmidt, H.; Kummerlöwe, C. *Polymer* **1999**, *40*, 5545.
96. Nojima, S.; Yamamoto, S.; Ashida, T. *Polymer Journal* **1995**, *27*, 673.
97. Balsamo, V.; von Gyldenfeldt, F.; Stadler, R. *Macromol. Chem. Phys.* **1996**, *197*, 3317.
98. Simon, Jr. C. G. *Journal of Microscopy* **2004**, *216*, 153.
99. Park, A.; Cima, L. G. *Journal of Biomedical Materials Research* **1996**, *31*, 117.
100. Washburn, N. R.; Yamada, K. M.; Simon, C. G., Jr.; Kennedy, S. B.; Amis, E. J. *Biomaterials* **2004**, *25*, 1215.
101. Simon, C. G., Jr.; Eidelman, N.; Kennedy, S. B.; Sehgal, A.; Khatri, C. A.; Washburn, N. R. *Biomaterials* **2005**, *6*, 6906.
102. Norman, J. J.; Desai, T. A. *Annals of Biomedical Engineering* **2006**, *37*, 89.
103. Flemming, R. G.; Murphy, C. J.; Abrams, G. A.; Goodman, S. L.; Nealey, P. F. *Biomaterials* **1999**, *20*, 573.
104. Lim, J. Y.; Donahue, H. J. *Tissue Engineering* **2007**, *13*, 1879.
105. Shin, H. *Biomaterials* **2007**, *28*, 126.
106. Bettinger, C. J.; Langer, R.; Borenstein, J. T. *Angew. Chem. Int. Ed. Engl.* **2009**, *48*,

5406.

107.Martinez, E.; Engel, E.; Planell, J. A.; Samitier, J. *Annals of Anatomy* **2009**, *191*, 126.

108.Lord, M. S.; Foss, M.; Besenbacher, F. *Nano Today* **2010**, *5*, 66.

109.Weiss, P. *J Exp Zool* **1945**, *100*, 353.

110.Clark, P.; Connolly, P.; Curtis, A. S. G.; Dow, J. A. T.; Wilkinson, C. D. W. *Development* **1990**, *108*, 635.

111.Clark, P.; Connolly, P.; Curtis, A. S. G.; Dow, J. A. T.; Wilkinson, C. D. W. *J Cell Sci* **1991**, *99*, 73.

112.Clark, P.; Connolly, P.; Moores, G. R. *J Cell Sci* **1992**, *103*, 287.

113.Lenhert, S.; Meier, M. B.; Meyer, U.; Chi, L.; Wiesmann, H. P. *Biomaterial* **2005**, *26*, 563.

114.Matsuzaka, K.; Walboomers, X. F.; Yoshinari, M.; Inoue, T.; Jansen, J. A. *Biomaterials* **2003**, *24*, 2711.

115.Yang, J. Y.; Ting, Y. C.; Lai, J. Y.; Liu, H. L.; Fang, H. W.; Tsai, W. B. *Journal of Biomedical Research Part A* **2009**, *90A*, 629.

116.Wang, J. H. C.; Grood, E. S.; Florer, J.; Wenstrup, R. *Journal of Biomechanics* **2000**, *33*, 729.

117.Zhu, B.; Lu, Q.; Yin, J.; Hu, J.; Wang, Z. *Tissue Engineering* **2005**, *11*, 825.

118.Fujita, S.; Ohshima, M.; Iwata, H. *Journal of the Royal Society Interface* **2010**, *6*, 269.

119. Groessner-Sreieber, B.; Tuan, R. S. *Journal of Cell Science* **1992**, *101*, 209.
120. Chehroudi, B.; McDonnell, D.; Brunette, D. M. *Journal of Biomedical Materials Research* **1997**, *34*, 279.
121. Chehroudi, B.; Ratkay, J.; Brunette, D. M. *Cells and Materials* **1992**, *2*, 89.
122. Matsuzaka, K.; Walboomers, X. F.; de Ruijter, J. E.; Jansen, J. A. *Biomaterials* **1999**, *20*, 1293.
123. Li, G.; Pittman, C. U. Jr. "Polyhedral oligomeric silsesquioxane (POSS) polymers, copolymers, and resin Nanocomposites" in *Macromolecules Containing Metal and metal-like Elements*, vol. 4, Group IVA Polymers, Abd-El-Aziz AS, Carraher CE Jr, Pittman CU Jr, Zeldin M. eds. chapter 5, pp. 79-131.
124. Li, G.; Wang, L.; Ni, H.; Pittman, C. U. Jr. *J Inorg Organomet P* **2001**, *11*, 123.
125. Schwab, J. J.; Lichtenhan, J. D. *Appl Organomet Chem* **1998**, *12*, 707.
126. Wu, J.; Mather, P. T. *J Macromol Sci, Part C: Polym Rev* **2009**, *49*, 25.
127. Kannan, R. Y.; Salacinski, H. J.; Butler, P. E.; Seifalian, A. M. *Acc Chem Res* **2005**, *38*, 879.
128. Li, G.; Wang, L.; Toghiani, H.; Daulton, T. L.; Koyama, K.; Pittman, C. U. Jr. *Macromolecules* **2001**, *34*, 8686.
129. Li, G.; Cho, H.; Wang, L.; Toghiani, H.; Pittman, C. U. Jr. *J Polym Sci Part A: Polym Chem* **2005**, *43*, 355.
130. Choi, J.; Yee, A. F.; Laine, R. M. *Macromolecules* **2003**, *36*, 5666.

- 131.Knight, P. T.; Lee, K. M.; Qin, H.; Mather, P. T. *Biomacromolecules* **2008**, *9*, 2458.
- 132.Liu, Y. R.; Huang, Y. D.; Liu, L. *Composite Sci Techn* **2007**, *67*, 2864.
- 133.Liu, Y. R.; Huang, Y. D.; Liu, L. *J Appl Polym Sci* **2008**, *110*, 2989.
- 134.Kopesky, E. T.; Boyes, S. G.; Treat, N.; Cohen, R. E.; McKinley, G. H. *Rheo Acta* **2006**, *45*, 971.
- 135.Kopesky, E. T.; Haddad, T. S.; McKinley, G. H.; Cohen, R. E. *Polymer* **2005**, *46*, 4743.
- 136.Kopesky, E. T.; McKinley, G. H.; Cohen, R. E. *Polymer* **2006**, *47*, 299.
- 137.Liu, Y.-L.; Tseng, M.-C.; Fangchiang, M.-H. *J Polym Sci Part A Polym Chem* **2008**, *46*, 5157.
- 138.Liu, Y.-L, Fangchiang, M. H. *J Mater Chem* **2009**, *19*, 3643.
- 139.Lee, J.-Y.; Lee, Y. M.; Bhattacharya, B.; Nho, Y.-C.; Park, J.-K. *J Solid State Electrochem* **2009**, *14*, 1445.
- 140.Bizet, S.; Galy, J.; Gérard, J.-F. *Polymer* **2006**, *47*, 8219.
- 141.Gao, F.; Tong, Y.; Schricker, S. R.; Culbertson, B. M. *Polym Adv Technol* **2001**, *12*, 355.
- 142.Kim, S. K.; Heo, S. J.; Koak, J. Y.; Lee, J. H.; Lee, Y. M.; Chung, D. J.; Lee, J. I.;
- 143.Fong, H.; Dickens, S. H.; Flaim, G. M. *Dent Mater* **2005**, *21*, 520.
- 144.Kannan, R. Y.; Salacinski, H. J.; Odlyha, M.; Butler, P. E.; Seifalian, A. M. *Biomaterials* **2006**, *27*, 1971.

- 145.Kannan, R. Y.; Salacinski, H. J.; Edirisinghe, M. J.; Hamilton, G.; Seifalian, A. M.
Biomaterials **2006**, *27*, 4618.
- 146.Kannan, R. Y.; Salacinski, H. J.; Groot, J. D.; Clatworthy, I.; Bozec, L.; Horton, M.;
Butler, P. E.; Seifalian, A. M. *Biomacromolecules* **2006**, *7*, 215.
- 147.Wang, W.; Guo, Y.; Otaigbe, J. U. *Polymer* **2009**, *50*, 5749.
- 148.Wang, K.; Cai, L.; Hao, F.; Xu, X.; Cui, M.; Wang, S. *Biomacromolecules* **2010**, *11*,
2748.
- 149.Isama, K.; Tsuchiya, T. *Biomaterials* **2003**, *24*, 3303.
- 150.Ikarashi, Y.; Tsuchiya, T.; Nakamura, A. *Biomaterials* **2000**, *21*, 1259.
- 151.Morrison, J. J.; Love, C. J.; Manson, B. W.; Shannon, I. J.; Morris, R. E. *J Mater*
Chem **2002**, *12*, 3208.
- 152.Qiu, Z.; Pan, H. *Compos Sci Technol* **2010**, *70*, 1089.
- 153.Kim, K.; Dean, D.; Mikos, A. G.; Fisher, J. P. *Biomacromolecules* **2009**, *10*, 1810.
- 154.Wutticharoenmongkol, P.; Pavasant, P.; Supaphol, P. *Biomacromolecules* **2007**, *8*,
2602.
- 155.Hronik-Tupaj, M.; Rice, W. L.; Cronin-Golomb, M.; Kaplan, D. L.; Georgakoudi, I.
BioMed Eng Online **2011**, *10*, 9.
- 156.Stein, G. S.; Lian, J. B.; Owen, T. A. *FASEB J* **1990**, *4*, 3111.
- 157.Mei, Y.; Saha, K.; Bogatyrew, S.; Yang, Y.; Hook, A. L.; Kalcioglu, Z. I.; Cho, S.-W.;
Mitalipova, M.; Pyzocha, N.; Rojas, F.; Van Vliet, K. J.; Davies, M. C.; Alexander, M.

- R.; Langer, R.; Jaenisch, R.; Anderson, D. G. *Nature Mater* **2010**, 9, 768.
- 158.Cai, L.; Guinn, A. S.; Wang, S. *Acta Biomater* **2011**, 7, 2185.
- 159.Georges, P. C.; Janmey, P. A. *J. Appl. Physiol.* **2005**, 98, 1547.
- 160.Levental, I.; Georges, P. C.; Janmey, P. A. *Soft Matter* **2007**, 3, 299.
- 161.Nemir, S.; West, J. L. *Ann. Biomed. Eng.* **2010**, 38, 2.
- 162.Brown, X. Q.; Ookawa, K.; Wong, J. Y. *Biomaterials* **2005**, 26, 3123.
- 163.Mirzadeh, H.; Shokrolahi, F.; Daliri, M. *J. Biomed. Mater. Res.* **2003**, 67A, 727.
- 164.Teixeira, A. I.; Ilkhanizadeh, S.; Wigenius, J. A.; Duckworth, J. K.; Inganäs, O.; Hermanson, O. *Biomaterials* **2009**, 30, 4567.
- 165.Zhang, K.; Simon, C. G. Jr.; Washburn, N. R.; Antonucci, J. M.; Lin-Gibson S. *Biomacromolecules* **2005**, 6, 1615.
- 166.Khan, Y.; Yaszemski, M. J.; Mikos, A. G.; Laurencin, C. T. *J. Bone Joint Surg. Am.* **2008**, 90A(S1), 36.
- 167.Chan-Park, M. B.; Shen, J. Y.; Cao, Y. Xiong, Y.; Liu, Y.; Rayatpisheh, S.; Kang, G. C.-W.; Greisler, H. P. *J. Biomed. Mater. Res.* **2009**, 88A, 1104.
- 168.Rubinstein, M.; Colby, R. H. *Polymer Physics*, Oxford: New York, 2003.
- 169.Wang, S.; Wang, S.-Q.; Halasa, A.; Hsu, W.-L. *Macromolecules* **2003**, 36, 5355.
- 170.Wang, S.; von Meerwall, E. D.; Wang, S.-Q.; Halasa, A.; Hsu, W.-L.; Zhou, J. P.; Quirk, R. P. *Macromolecules* **2004**, 37, 1641.
- 171.Wang, S.; Elkasabi, Y.; Wang, S.-Q. *Macromolecules* **2005**, 38, 125.

- 172.Brandrup, J.; Immergut, E. H. Eds. *Polymer Handbook*, 3rd ed.; Wiley: New York, 1989.
- 173.Wang, K.; Wang, S. *Abstr. Pap. Am. Chem. Soc.* **2010**, 238, PMSE-177.
- 174.Khatiwala, C. B.; Peyton, S. R.; Putnam, A. J. *Am. J. Physiol. Cell Physiol.* **2006**, 290, 1640.
- 175.Marklein, R. A.; Burdick, J. A. *Soft Matter* **2010**, 6, 136.
- 176.Zaari, N.; Rajagopalan, P.; Kim, S. K.; Engler, A. J.; Wong, J. Y. *Adv. Mater.* **2004**, 16, 2133.
- 177.Lo, C.-M.; Wang, H.-B.; Dembo, M.; Wang, Y.-L. *Biophys. J.* **2000**, 79, 144.
- 178.Wong, J. Y.; Velasco, A.; Rajagopalan, P.; Pham, Q. *Langmuir* **2003**, 19, 1908.
- 179.Peyton, S. R.; Kim, P. D.; Ghajar, C. M.; Seliktar, D.; Putnam, A. J. *Biomaterials* **2008**, 29, 2597-2607.
- 180.Engler, A.; Bacakova, L.; Newman, C.; Hategan, A.; Griffin, M.; Discher, D. *Biophys. J.* **2004**, 86, 617.
- 181.Mousallem, M. D.; Olenych, S. G.; Scott, S. L.; Keller, T. C. S. III; Schlenoff, J. B. *Biomacromolecules* **2009**, 10, 3062.
- 182.McDaniel, D. P.; Shaw, G. A.; Elliott, J. T.; Bhadriraju, K.; Meuse, C.; Chung, K.-H.; Plant, A. L. *Biophys. J.* **2007**, 92, 1759.
- 183.Bhatia, Q. S.; Pan, D. H.-K.; Koberstein, J. T. *Macromolecules* **1988**, 21, 2166.
- 184.Nishino, T.; Matsumoto, T.; Nakamae, K. *Polym. Eng. Sci.* **2000**, 40, 336.

- 185.Hoffman, J. D.; Weeks, J. J. *J. Res. Natl. Bur. Stand. Sect. A* **1962**, 66A, 3.
- 186.Flory, P. J. *Principles of Polymer Chemistry*; Cornell University Press: New York, 1953.
- 187.Nishi, T.; Wang, T. T. *Macromolecules* **1975**, 8, 909.
- 188.Ziska, J. J.; Barlow, J. W.; Paul, D. R. *Polymer* **1981**, 22, 918.
- 189.Meng, Y.; Li, H.; Huo, H.; Jiang, S.; An, L. *Journal of Applied Polymer Science* **2007**, 8, 615.
- 190.Ma, D.; Cai, H.; Zhang, J.; Luo, X. *Macromol. Chem. Phys.* **1999**, 200, 2040.
- 191.Rechendorff, K.; Hovgaard, M. B.; Foss, M.; Zhdanov, V. P.; Besenbacher, F. *Langmuir* **2006**, 22, 10885.
- 192.Scopelliti, P. E.; Borgonovo, A.; Indrieri, M.; Giorgetti, L.; Bongiorno, G.; Carbone, R.; Podesta, A.; Milani, P. *PLoS One* **2010**, 5, e11862.
- 193.Gumbiner, B. M. *Cell* **1996**, 84, 345.
- 194.Wang, Z.; Wang, X.; Yu, D.; Jiang, B. *Polymer* **1997**, 38, 5897.
- 195.Phillips, P. J.; Rensch, G. Y.; Taylor, K. D. *J. Polym. Sci.: Part B: Polym. Phys.* **1987**, 25, 1725.
- 196.Wang, K.; Cai, L.; Wang, S. *Abstr. Pap. Am. Chem. Soc.* **2010**, 103, PMSE-524.
- 197.Gentile, F.; Tirinato, L.; Battista, E.; Causa, F.; Liberale, C.; di Fabrizio, E. M.; Decuzzi, P. *Biomaterials* **2010**, 28, 7205.
- 198.Aubin, H.; Nichol, J. W.; Hutson, C. B.; Bae, H.; Sieminski, A. L.; Cropek, D. M.;

- Akhyari, P.; Khademhosseini. *Biomaterials* **2010**, *31*, 6941.
- 199.Dalby, M. J.; Riehle, M. O.; Yarwood, S. J.; Wilkinson, C. D.; Curtis, A. S. *Experimental Cell Research* 2003, 284, 274.
- 200.Gerecht, S.; Bettinger, C. J.; Zhang, Z.; Borenstein, J. T.; Vunjak-Novakovic, G.; Langer, R. *Biomaterials* **2007**, *28*, 4068.
- 201.Davidson, P. M.; Özçelik, H.; Hasirci, V.; Reiter, G.; Anselme, K. *Advanced Materials* **2009**, *21*, 3586.
- 202.Moriguchi, T.; Yano, K.; Nakagawa, S.; Kaji, F. *Journal of Colloid and Interface Science* **2003**, *260*, 19.
- 203.Ishaug, S. L.; Payne, R. G.; Yaszemski, M. J.; Thomas, B.; Aufdemorte, T. B.; Bizios, R.; Mikes, A. G. *Biotechnology and Bioengineering* **1996**, *50*, 443.
- 204.Lamers, E.; Walboomers, X. F.; Domanski, M.; te Riet, J.; van Delft, F. C.; Luttge, R.; Winnubst, L. A.; Gardeniers, H. J.; Jansen, J. A. *Biomaterials* **2010**, *31*, 3307.
- 205.Li, F.; Li, B.; Wang, Q. M.; Wang, J. H. C. *Cell Motility and the Cytoskeleton* **2008**, *65*, 332.
- 206.Wang, J. H. C.; Jia, F.; Gilbert, T. W.; Woo, S. L. Y. *Journal of Biomechanics* **2003**, *36*, 97.
- 207.Tang, J.; Peng, R.; Ding, J. *Biomaterials* **2010**, *31*, 2470.
- 208.Steedman, M. R.; Tao, S. L.; Klassen, H.; Desai, T. A. *Biomed Microdevices* **2010**, *12*, 363.

- 209.Yim, E. K. F.; Pang, S. W.; Leong, K. W. *Experimental Cell Research* **2007**, *313*, 1820.
- 210.Pijnappels, D. A.; SchaliJ, M. J.; Ramkisoensing, A. A.; van Tuyn, J.; de Vries, A. A. F.; van der Laarse, A.; Ypey, D. L.; Atsma, D. E. *Circ Res* **2008**, *103*, 167.

Vita

Kan Wang was born in 1982 at Anshan, Liaoning, China. He got his Bachelor's degree in 2005, majoring in Materials Science and Engineering, from Beijing University of Chemical Technology (BUCT), Beijing, China. Then he continued his graduate study at BUCT for another two years, and got his Master's degree in Materials Science. In January of 2008, he enrolled in the doctoral program of Polymer Engineering at the University of Tennessee, Knoxville.