

Design, Fabrication and Characterization of Biodegradable Scaffolds for Tissue Engineering and Regenerative Medicine Applications

by

Muhammad Anwaar Nazeer

A Dissertation Submitted to the
Graduate School of Sciences and Engineering
in Partial Fulfillment of the Requirements for
the Degree of
Doctor of Philosophy
in
Biomedical Sciences and Engineering



**KOÇ
UNIVERSITY**

August 2018

Design, Fabrication and Characterization of Biodegradable Scaffolds for Tissue Engineering and Regenerative Medicine Applications

Koç University

Graduate School of Sciences and Engineering

This is to certify that I have examined this copy of a doctoral dissertation by

Muhammad Anwaar Nazeer

and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Committee Members:

Prof. Dr. İskender Yılgör

Prof. Dr. Halil Kavaklı

Assoc. Prof. Dr. Funda Acar Yağcı

Assoc. Prof. Dr. Pınar Yılgör Huri

Assoc. Prof. Dr. Kadriye Tuzlakoğlu

Date: August 29, 2018

ABSTRACT

Biocompatible and biodegradable scaffolds for tissue engineering and regenerative medicine applications were developed. Bone and skin tissue engineering applications were focused throughout the study. Biomaterials such as hydroxyapatite (HA), chitosan (CS) and poly(lactic acid) (PLA) were used to fabricate scaffolds for potential bone regeneration applications. HA was synthesized through different production routes via sol-gel process. The process was critically analyzed and evaluated for synthesis of pure HA. Nano-composite scaffolds of CS/HA were fabricated through simple solvent casting method. Selection of proper solvent media (formic acid, a green solvent) produced stable and well defined dispersion of HA that resulted in intercalated structural composites. Furthermore, 3D PLA scaffolds were developed through fused deposition modeling and CS/HA were incorporated in these scaffolds. Formic acid enabled embedding of CS/HA through chemical etching of PLA surfaces. These scaffolds were analyzed *in-vitro* by seeding U2-OS human osteosarcoma cells. Scaffolds modified with CS/HA showed higher cell proliferation as compared to unmodified ones. The scaffolds developed in this study show great potential for use as substrates for bone tissue engineering applications.

For skin tissue engineering applications, polycaprolactone (PCL) and the blend of a polyurethane (PU) prepared through stoichiometric reaction of PCL ($M_n=2000$ g/mol) and 1,6-hexamethylene diisocyanate (HDI) and silk fibroin (SF) were used. PCL was synthesized through ring opening polymerization of ϵ -caprolactone in the presence of T-9 catalyst and 1,2-bis(3-aminopropoxy)ethane as initiator. SF was obtained by degumming of silk cocoons through a novel process developed in this study and subsequent dissolution. Nano-fibrous scaffolds of PCL and PU with and without different amounts of SF were prepared through a “green” electrospinning process in formic acid, which is a low toxicity Q3C class 3 solvent, rather than commonly used class 2 solvents such as tetrahydrofuran (THF) and dimethylformamide (DMF). Successful addition of SF and its effect on these scaffolds was analyzed through various characterization techniques. *In-vitro* analysis was done by seeding Human BJ and NIH/3T3-Mouse embryo fibroblast cells on PCL, PCL/SF and PU, PU/SF scaffolds respectively. ATP and MTT cytotoxicity analyses revealed significant enhanced proliferation of fibroblast cells as compared to pristine PCL and PU

scaffolds. We believe these novel composite scaffolds with good mechanical properties and bioactivity can be used for skin tissue engineering applications.

Keywords: Biodegradable scaffolds, skin tissue engineering, bone tissue engineering applications



ÖZETÇE

Bu çalışmada doku mühendisliği uygulamaları için vücutta çözünebilir yapılar geliştirilmiştir. Ana tema olarak kemik ve deri doku uygulamalarına odaklanılmıştır. Hidroksiapatit (HA), kitosan (CS) ve poli(laktik asit) (PLA) gibi biyouyumlu malzemeler doku geliştirme çalışmaları için kullanılmıştır. Saf HA sol-jel metodu kullanılarak değişik yöntemler ile üretilmiş ve karakterize edilmiştir. Nano boyutta geliştirilen kitosan-hidroksiapatit (CS/HA) karışımları çözücü uçurma metodu ile elde edilmiştir. Bu amaç için en uygun çözücünün formik asit olduğu görülmüştür. Bu çözücü içerisinde HA homojen bir şekilde dağılmış ve kitosan zincirleri arasına girerek değişik morfolojiler meydana getirmiştir. Ayrıca, üç boyutlu baskı yöntemi ile PLA doku iskeleleri geliştirilmiştir. Formik asit kullanarak hazırlanan CS/HA karışımları, 3 boyutlu PLA malzemelerinin yüzeyine kaplanmıştır. Üretilen doku malzemelerinin üzerlerinde U2-OS insan kemik hücreleri büyütülmüştür. CS/HA eklenmesi, hücrelerin doku malzemesi üzerinde büyümelerini geliştirmiştir. Üretilen malzemelerin kemik doku mühendisliği uygulamalarında kullanılmaya uygun olduğu tespit edilmiştir.

Doku mühendisliği uygulamaları için yüksek molekül ağırlıklı polikaprolakton (PCL), PCL ($M_n=2000$ g/mol) içeren bir poliüretan (PU) ve ipek (SF) malzemeleri kullanılmıştır. Yüksek molekül ağırlıklı PCL epsilon-kaprolakton monomerinin polimerizasyonu ile üretilmiştir. PU ise PCL-2000 oligomeri ve 1,6-hekzametilen diizosiyanat'ın (HDI) stokiyometrik (1/1 mol) reaksiyonu ile üretilmiştir. İpek lifleri ipek böceği kozasından ekstrakte ederek elde edilmiştir. Bu yöntemde daha yaygın olan THF ve DMF çözücüleri yerine daha az zararlı bir çözücü olan formik asit kullanılarak yeşil elektro-eğirme yöntemi ile nanofiber yapılar elde edilmiştir. Üretilen malzemeler değişik metotlarla karakterize edilmiştir. Hücre çalışmaları insan BJ ve fare NIH/3T3 hücreleri kullanılarak gerçekleştirilmiştir. ATP ve MTT sitotoksitesite testleri sonucunda hücrelerin PCL ve PU malzemeleri ile karşılaştırıldığında diğerlerine göre daha çok büyüdükleri gözlemlenmiştir. Elde edilen malzemelerin deri doku mühendisliği uygulamalarında kullanılması için gerekli olan mekanik ve biyolojik özelliklere sahip oldukları gözlemlenmiştir.

Anahtar Kelimeler: Vücutta çözünebilir doku malzemeleri, deri doku mühendisliđi, kemik doku mühendisliđi uygulamaları



ACKNOWLEDGMENTS

Many individuals from KOÇ Family have played their role to make my dream of PhD come true and I would like to thank for their help and support throughout of my 5 years here in KOÇ University. First of all, I would like to acknowledge kind supervision and encouraging support from my advisor Prof. Dr. İskender Yılgör and Emel Yılgör throughout of my PhD tenure. Being a part of Polymer Research Lab will be a great asset for me.

I would like to concede the support of faculty members of KOÇ University who tried to enrich my knowledge during coursework here. I also owe a debt of gratitude to my thesis monitoring committee members: Prof. Dr. Halil Kavaklı and Assoc. Prof. Dr. Funda Acar Yağcı for their kind support and valuable suggestions to improve my work.

I would like to thank my friends and colleagues, Özgün Can Önder, Ipek Atay, Çağla Söz, Armen Yıldırım, Melis Peniç from polymer lab and Dr. Mustafa Barış Yağcı, Dr. Ceren Yılmaz Akkaya, Cansu Yildirim from KUYTAM, who helped me always.

On this great occasion, I would like to remember my father, Nazeer Ahmad (late) and mother, Hameeda Begum (late) who always supported me in my life and I am sure their prayers are still with me today that made this happen to complete my PhD. I would like to thank my wife, Syeda Rubab Batool, who always supports me through thick and thin of life's triumphs.

At the end, I would like to thank Pakistan Higher Education Commission, who offered financial support to peruse for PhD through HRDI-UESTP (Faculty development program for engineering universities of Pakistan) program.

TABLE OF CONTENTS

List of Tables	xiii
List of Figures	xiv
Nomenclature	xvii
1 Introduction and background	19
1.1 Tissue engineering and regenerative medicine	19
1.2 Tissue engineering applications	20
1.3 Biomaterials	22
1.3.1 Hydroxyapatite	23
1.3.2 Chitosan	24
1.3.3 Silk fibroin	25
1.3.4 Poly(lactic acid)	27
1.3.5 Polycaprolactone.....	27
1.3.6 Polyurethane	28
1.3.7 Hybrid materials and composites.....	29
1.4 Fabrication techniques	31
1.4.1 Additive manufacturing techniques.....	31
1.4.2 Electrospinning technique	32
2 Hydroxyapatite synthesis	34
2.1 Summary	34
2.2 Materials and methods	35
2.2.1 Materials	35
2.2.2 Methods	35
2.2.2.1 Hydroxyapatite synthesis	35

2.2.2.2	Characterization techniques	36
2.3	Results and discussion	37
2.3.1	X-ray diffraction analysis	37
2.3.2	ATR-FTIR Analysis	39
2.3.3	Raman analysis	40
2.3.4	Surface analysis	43
2.3.5	Effect of solvent on physical and chemical properties	44
2.4	Conclusions	46
3	Intercalated chitosan/hydroxyapatite nano-composites.....	47
3.1	Summary	47
3.2	Materials and methods	48
3.2.1	Materials	48
3.2.2	Methods	48
3.2.3	Hydroxyapatite synthesis.....	48
3.2.4	Chitosan/hydroxyapatite (CS/HA) nanocomposites development	48
3.2.5	Characterization Techniques	49
3.3	Results and Discussion.....	50
3.3.1	Characterization of hydroxyapatite.....	50
3.3.2	Characterization of chitosan/hydroxyapatite (CS/HA) nanocomposites	51
3.3.2.1	ATR-FTIR and XRD analysis of CS/HA nanocomposites	51
3.3.2.2	Cross-sectional analysis	53
3.3.2.3	Surface analysis	54
3.3.2.4	Thermal Degradation Studies	56
3.3.2.5	Stress-strain analysis	58
3.4	Conclusions	58
4	PLA/CS/HA 3D composite scaffolds development	60

4.1	Summary	60
4.2	Materials and methods	62
4.2.1	Materials	62
4.2.2	Methods	62
4.2.2.1	Hydroxyapatite synthesis	62
4.2.2.2	3D printed PLA and PLA/CS/HA composite scaffolds development	62
4.2.2.3	Characterization Techniques	63
4.3	Results and Discussion.....	64
4.3.1	Characterization of PLA/CS/HA composites	64
4.3.1.1	ATR-FTIR analysis	64
4.3.1.2	Surface analysis	65
4.3.1.3	XRD analysis.....	68
4.3.1.4	Weight loss behavior of scaffolds in DMEM medium.....	69
4.3.1.5	In vitro cell viability	71
4.4	Conclusions	72
5	Silk cocoons degumming and silk fibroin processing.....	73
5.1	Summary	73
5.2	Materials and Methods.....	75
5.2.1	Materials	75
5.2.2	Methods	75
5.2.2.1	Silk cocoons degumming	75
5.2.2.2	Dissolution of degummed fibers	75
5.2.2.3	Preparation of silk fibroin films	76
5.2.3	Characterization techniques.....	76
5.3	Results and discussions.....	77
5.3.1	Characterization of degummed silk fibers.....	77

5.3.1.1	SEM analysis	77
5.3.1.2	ATR-FTIR analysis	78
5.3.1.3	X-rays diffraction analysis	79
5.3.2	Characterization of silk fibroin films.....	80
5.3.2.1	ATR-FTIR analysis	81
5.3.2.2	X-ray diffraction analysis.....	84
5.3.2.3	AFM analysis	86
5.3.2.4	Water contact angle measurement.....	88
5.4	Conclusions.....	89
6	Fabrication of PCL/SF nano-fibrous scaffolds	91
6.1	Summary	91
6.2	Materials and Methods.....	92
6.2.1	Materials	92
6.2.2	Methods	92
6.2.2.1	PCL synthesis through ring-opening polymerization.....	92
6.2.2.2	PCL/SF solutions and their electrospinning.....	93
6.2.2.3	Characterization techniques	94
6.3	Results and discussions.....	95
6.3.1	ATR-FTIR analysis	95
6.3.2	Surface analysis	96
6.3.3	Degradation studies	98
6.3.4	Mechanical properties of scaffolds.....	99
6.3.5	In vitro cell viability analysis	100
6.4	Conclusions.....	103
7	Fabrication of PU/SF nano-fibrous scaffolds	104
7.1	Summary	104

7.2	Materials and methods	105
7.2.1	Materials	105
7.2.2	Methods	105
7.2.2.1	Polyurethane synthesis	105
7.2.2.2	Polyurethane/silk fibroin nano-fibrous scaffolds development.....	106
7.2.2.3	Characterization techniques	107
7.3	Results and discussion	107
7.3.1	ATR-FTIR analysis	107
7.3.2	Surface analysis	109
7.3.3	Degradation analysis.....	111
7.3.4	Mechanical properties of scaffolds	112
7.3.5	In-vitro cells viability analysis.....	113
7.4	Conclusions.....	115
8	Conclusions and future directions.....	117
9	Bibliography	120
10	Appendices.....	135
	Appendix-I.....	135
	Appendix-II.....	136
	Appendix-III	137
	Appendix-IV	138
	Appendix-V	139
	Appendix-VI.....	140

LIST OF TABLES

Table 2.1. XRD analysis of Hydroxyapatite and β -Calcium pyrophosphate	38
Table 2.2. Influence of the dielectric constant of the solvent mixture on BET, XRF and XRD analysis of calcium phosphate synthesized (a=k value of 0.89, b=k value of 1)	45
Table 3.1. Characteristic IR bands positions for chitosan, hydroxyapatite, β -tricalcium phosphate and calcium pyrophosphate [199, 200]	52
Table 4.1. Samples prepared, their coding and description.....	63
Table 5.1. Different silk conformations content of films (*Before methanol treatment, **After methanol treatment)	83
Table 6.1. PCL/SF concentrations, electrospinning conditions and average fiber diameter.....	94
Table 7.1. Sample coding, solution concentration, spinning parameters and fibers diameter	106

LIST OF FIGURES

Figure 1.1. Tissue engineering concept from cell isolation/culturing to seeding, tissue formation and implantation.....	19
Figure 1.2. Primary structure of the heavy chain of <i>Bombyx mori</i> silk fibroin.....	25
Figure 1.3. Schematic demonstration of segmented TPUs	29
Figure 1.4. Schematic representation of basic fused deposition modelling process	31
Figure 1.5. Basic electrospinning setup for nano-fibrous scaffolds development	33
Figure 2.1. Schematic representation of the reaction schemes followed in calcium phosphate synthesis through sol-gel process.....	36
Figure 2.2. XRD analysis of calcined calcium phosphate powders synthesized by using water, THF and DMF as the reaction co-solvent.....	37
Figure 2.3. ATR-FTIR analysis for synthesized calcium phosphate powders obtained through different reaction schemes.....	39
Figure 2.4. Raman analysis of calcium phosphate powders obtained from different reaction schemes	41
Figure 2.5. Raman mapping of synthesized calcium phosphate phases (scale bar=20 μm), HA (red), β -CPF (green).....	42
Figure 2.6. FESEM analysis of calcium phosphate powders synthesized in different solvent media	43
Figure 3.1. (a) ATR-FTIR spectra (b) XRD analysis of hydroxyapatite; after 1 h heat treatment at 500 $^{\circ}\text{C}$ (HA-500) and 1100 $^{\circ}\text{C}$ (HA-1100).....	50
Figure 3.2. ATR-FTIR spectra of; (a) hydroxyapatite, chitosan and their composites; (b) de-convoluted spectrum of CS/HA-20; (c) XRD analysis of HA-500, chitosan and their composites	51
Figure 3.3. Cross-sectional SEM images of (a) CS/HA-0, (b) CS/HA-5, (c) CS/HA-10, (d) CS/HA-20; EDX mapping for Calcium and Phosphorous of (e) CS/HA-5, (f) CS/HA-10, (g) CS/HA-20	

and (h) schematic description of the presence of a layered morphology in CS/HA nanocomposites	53
Figure 3.4. SEM surface images of (a) CS, (b) CS/HA-5, (c) CS/HA-10 and (d) CS/HA-20 nanocomposites	54
Figure 3.5. 5x5 μm^2 AFM phase and height images of CS and CS/HA nanocomposite films	55
Figure 3.6. (a) TGA curves for HA, chitosan and CS/HA nanocomposites; (b) ATR-FTIR spectra for HA-1100 and TGA residues of CS/HA nanocomposites	56
Figure 3.7. SEM analysis of residues obtained after thermal degradation of composites.....	57
Figure 3.8. Stress-strain analysis of CS and CS/HA nanocomposites	58
Figure 4.1. Schematic representation of PLA/CS/HA composite scaffolds development process.....	61
Figure 4.2. ATR-FTIR analysis of PLA, CS, HA and their composites.....	64
Figure 4.3. SEM analysis of PLA, PLA+CS, PLA+CS/HA-10 and PLA+CS/HA-20 scaffolds..	66
Figure 4.4. SE images, EDX mapping and spectra for: PLA+CS/HA-10 (a, b, c) and PLA+CS/HA-20 (d, e, f).....	67
Figure 4.5. Static water contact angle measurement of PLA, PLA+CS, PLA+CS/HA-10 and PLA+CS/HA-20	68
Figure 4.6. XRD diffractograms of PLA, CS, HA and their composites	69
Figure 4.7. Weight loss behavior of PLA and PLA/CS and PLA/CS/HA scaffolds.....	70
Figure 4.8. MTT analysis of scaffolds after 1 st and 4 th day of osteosarcoma cell seeding	71
Figure 5.1. Schematic representation of the process steps used for degumming and recovery of silk fibroins and preparation of film from silk fibroin	74
Figure 5.2. Surface SEM images of cocoons and degummed fibers: (a) cocoon fibers, (b) Carbonate-45, (c) Bicarbonate-45, (d) Ammonia-45, (e) Ammonia-60, and (f) Ammonia-90	77
Figure 5.3. Comparative FTIR spectra of cocoons and degummed fibers obtained by using various processes	79
Figure 5.4. XRD analysis of cocoons and degummed fibers	80
Figure 5.5. ATR-FTIR analysis of silk fibroin films before and after methanol treatment	81

Figure 5.6. XRD diffractograms of cocoons and regenerated silk fibroin film: (a) before (b) after methanol treatment.....	85
Figure 5.7(a). Phase images of SF films obtained through different processes before methanol/water treatment (Scale bar=2 μ m).....	86
Figure 5.8. Water contact angle on silk fibroin films before and after methanol treatment.....	89
Figure 6.1. Schematic representation of PCL synthesis through ROP.....	93
Figure 6.2. ATR-FTIR analysis of PCL and PCL/SF composite scaffolds having different ratios of SF	95
Figure 6.3. SEM analysis of PCL (a, d) and PCL/SF-20 (c, d) and PCL/SF-40 (e, f) nano-fibrous scaffolds.	96
Figure 6.4. Water contact angle measurements for spin coated and electro-spun scaffolds	97
Figure 6.5. Degradation studies of PCL and PCL/SF nano-fibrous composite scaffolds	99
Figure 6.6. Mechanical properties comparison of PCL, PCL/SF and human skin: a) ultimate tensile strength, b) elongation at break and c) elastic modulus	100
Figure 6.7. ATP release in cell seeding analysis measured through luminescence (n=5).....	101
Figure 6.8. Surface morphology of cells on scaffolds: PCL (a, b); PCL/SF-20 (c, d) and PCL/SF-40 (e, f).	102
Figure 7.1. Schematic representation of PCL2k/HDI (1:1) polyurethane synthesis	106
Figure 7.2. ATR-FTIR analysis of SF powder, PU and PU/SF nano-fibrous scaffolds.....	108
Figure 7.3. SEM analysis of nano-fibrous scaffolds: (a, b) PU, (c, d) PU/SF-10 and (e, f) PU/SF-20.....	109
Figure 7.4. Water contact angle measurements on spin coated and electro-spun scaffolds.....	110
Figure 7.5. Remaining weight of nano-fibrous scaffolds against time	111
Figure 7.6. Mechanical properties comparison of PU, PU/SF and human skin: a) ultimate tensile strength, b) elongation at break and c) elastic modulus	112
Figure 7.7. MTT analysis of scaffolds after 1 st and 4 th day of osteosarcoma cell seeding	114
Figure 7.8. SEM analysis of scaffolds after cells seeding: PU (a, b, c), PU/SF-10 (d, e, f) and PU/SF-20 (g, h, i).	115

NOMENCLATURE

3D	Three Dimensional
AFM	Atomic force microscopy
ATR-FTIR	Attenuated total reflection-Fourier transform infrared spectroscopy
BDI	1,4-Butylene diisocyanate
BMMSCs	Bone marrow mesenchymal stem cells
CA	Contact angle
CNTH	Calcium nitrate tetrahydrate
CP	Calcium phosphate
CS	Chitosan
DAHP	Diammonium hydrogen phosphate
DLS	Dynamic light scattering
DMF	Dimethylformamide
DSC	Differential scanning calorimetry
ECM	Extra cellular matrix
EM	Elastic modulus
FA	Formic acid
FDA	Food and drug administration
FDM	Fused deposition modelling
FESEM	Field emission scanning electron microscopy
GPC	Gel permeation chromatography
HA	Hydroxyapatite
HDI	1,6-Hexamethylene diisocyanate

HMDI	4,4'-Methylenebis(cyclohexyl isocyanate)
IPDI	Isophorone diisocyanate
M _n	Number-average molecular weight
MSCs	Mesenchymal stem cells
M _w	Weight-average molecular weight
PCL	Polycaprolactone
PLA	Poly(lactic acid)
PU	Polyurethane
PVA	Poly(vinyl alcohol)
ROP	Ring opening polymerization
SE	Secondary electron
SEM	Scanning electron microscopy
SF	Silk fibroin
SFF	Solid freeform fabrication
TDI	Toluene diisocyanate
TGA	Thermogravimetric analysis
THF	Tetrahydrofuran
TPE	Thermoplastic elastomers
UTM	Ultimate tensile strength
XRD	X-ray diffraction
β-CPF	β-Calcium pyrophosphate
β-TCP	β-Tricalcium phosphate

CHAPTER 1

INTRODUCTION AND BACKGROUND

1.1 Tissue engineering and regenerative medicine

In tissue engineering, extra cellular matrix (ECM), which acts as a scaffold provide structural support, shape and proper surface for the cells to accommodate and guide in a tissue/organ. For effective functioning they require some physical and biological characteristics as well [1].

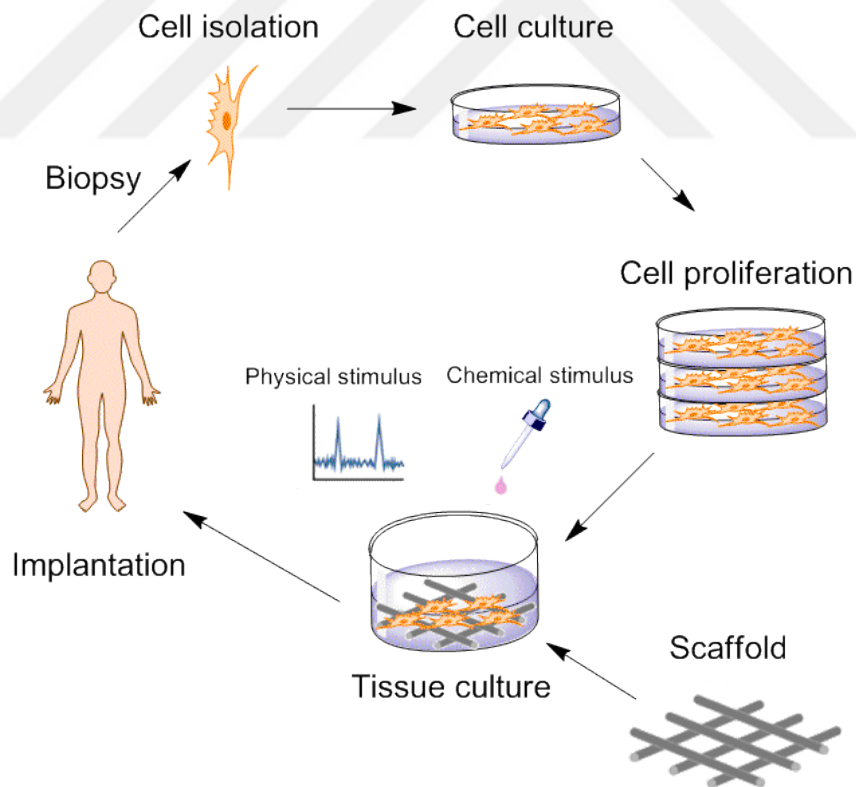


Figure 1.1. Tissue engineering concept from cell isolation/culturing to seeding, tissue formation and implantation

Proper structural support is needed for cell attachment, their migration, proliferation and differentiation. Mechanical properties of these scaffolds should be compatible with surrounding tissues or organs. Scaffold's surface topography should favor proper binding of the cells and porosity is needed for the diffusion of nutrients and for vascularization [2, 3]. In natural ECM, in addition to mechanical support, some important signaling molecules and regulatory functions are also present. Through proper incorporation of hormones and growth factors specifically designed for intended cell microenvironment, natural ECM can be mimicked [4]. Tissue engineering concept from cell isolation/culturing to cell seeding on porous scaffolds and ultimately tissue formation is schematically illustrated in Figure 1.1. So, the mechanical integrity and creation of a proper environment for cells are the key requirements/functions for well-designed biomaterials to mimic cell's natural ECM.

1.2 Tissue engineering applications

Based on the results generated from a large number of studies focusing on different applications of tissue engineering, it has been concluded that biomaterials utilized to function as ECM should not be considered to provide only structural support but much more. Since ECM has to interact with cells and biological environments, their interaction should be studied in depth. Over three decades, results obtained from research which focused on a wide range of applications it has been demonstrated that tissue engineering has tremendous potential for bone, skin, cartilage, liver, kidney, muscle and nerve regenerative medicine applications [5].

Treatment of skeletal defects remained a great challenge for many reconstructive surgical procedures. Currently autogenous bone is considered best option but a huge gap between supply and demand of autogenous bones and morbidity risk has compelled surgeons to look at alternative options of bone materials. Engineered graft materials through progenitor cells harvesting *in-vitro* and then clinical applications of graft materials *in-vivo* has been successfully performed. Combination of stem cells/progenitor cells, growth factors and scaffolds has also been used [6, 7]. Stem cells like mesenchymal stem cells (MSCs) have capability of self-renewing and differentiation into different lineages. Among MSCs, bone marrow mesenchymal stem cells (BMMSCs) showed great potential by proliferating and by showing osteogenic capability as demonstrated through various

studies [8]. Higher *in-vivo* bone regeneration was revealed in conjunction with MSCs as compared to acellular scaffolds [9] and in the presence of growth factors [10]. So, in bone tissue engineering, a suitable scaffold is developed that provides osteo-conductive environment to stem/progenitor cells to make them differentiate or proliferate. Cells are able to migrate to on ECM to defect site and create their own vascularization for proper infiltration and growth. Scaffolds can also be used to deliver biological treatments to provide osteogenic environment to cells to treat bone defects [11].

Skin injuries and their cure also provides a critical challenge due to the involvement of many complications in wound healing process. Low-cost dressing materials providing appropriate environment simultaneously is a great challenge for researchers. Inappropriate conditions during wound healing process can lead to serious problems such as scar formation and microbial attack and infection [12]. Consistent supply of nutrients, enzymes, oxygen and cells to the wounded region is required for fast healing process. Scaffold should provide a 3D environment mimicking natural ECM to the dermal cells in order to grow and regenerate into a new skin [13]. Essentially, skin has a two layered structure, one is epidermis and the other one dermis. Epidermis, consists of highly interconnected and permeable keratinocytes layers. It provides protection against external perils and helps to prevent water loss. On the other hand, dermis, a relatively elastic and vascularized tissue, provides nutrients and energy to epidermis. The hypodermis, under dermis, is a loose connective tissue having hair roots and numerous cells [14, 15]. Biocompatibility and biodegradability of natural polymers like collagen make them suitable candidates for skin grafts development [16]. However, low cost synthetic polymers such as degradable polyurethane and PCL can also be used as skin substitutes due to their biocompatibility and biodegradability [17, 18].

Articular cartilage, a tissue found between bones, can tolerate shear and compressive forces over the course of years due to low friction and high load bearing capabilities. This is due to the unique composition of ECM found in cartilages [19]. The repetitive/continuous cyclic loading may emasculate the collagen fibril's that leads to osteoarthritis [20]. Scaffolds manufactured specially for cartilage tissue engineering can be a better option for treating osteoarthritis than using MSCs to grow fibrous structures. Material and scaffolds design versatility enables to fabricate biomimetic, 3D and gradient structures that are key requirements for connective tissues [21].

Blood vessel replacement/repairing is being carried out in clinical trials for dialysis access and pediatric cardiovascular surgeries [22, 23]. Tissue engineered blood vessels have been developed through various techniques by utilizing natural and synthetic polymers and cell seeding as well [24, 25].

1.3 Biomaterials

Biomaterials are materials of natural or synthetic origin that are used to direct, appendage or provide substitute to the functions of native tissues. These materials are termed as biocompatible when they elude minimal biological response after coming in contact with biological tissues [26]. During the past several decades, due to ongoing demands and developments in tissue engineering, regenerative medicine and controlled drug release applications, researchers have been trying to make better biomaterials with specific combination of performance criteria. These biomaterials are designed keeping in view the required properties mainly non-cytotoxicity, bioactivity and biodegradation. In general, naturally available biomaterials remain to be the first choice due to their much enhanced characteristics, where they can be used as is or through suitable modifications. Due to lower cost associated with and better control on properties, synthetic materials are also being utilized. Since the biomaterials provide structural support in form of ECM, the main focus of research especially in synthetic materials is to tune their properties according to native tissues. For degradable materials, the concentration of degraded products should also be in a limit [27].

In this thesis, several synthetic or natural polymers were investigated as biomaterials for their possible use in bone and skin tissue engineering applications. Novel materials and synthesis routes were opted for developing biodegradable scaffolds. Hydroxyapatite, poly(lactic acid), and chitosan were explored for bone tissue engineering applications while silk fibroin, polycaprolactone and a segmented thermoplastic polyurethane were used for developing biodegradable soft tissues for skin regeneration applications. Non-cytotoxicity of synthesized materials and scaffolds was ensured by *in-vitro* cell viability assays.

1.3.1 *Hydroxyapatite*

Hydroxyapatite (HA) $[\text{Ca}_5(\text{PO}_4)_3\text{OH}]$ also denoted as $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ in order to show the hexagonal crystal unit cell consisting of two entities, constitutes about 70% by weight of the human bone. HA has become an important material in the studies of biomaterials and tissue engineering, especially in bone regeneration applications because of its high bioactivity and osteo-conductive characteristics [28-30]. HA, a natural mineral found in rocks is also a major constituent of the teeth, as well as bones [31, 32]. Even though, the autologous grafts provide the desired characteristics, they are also associated with some drawbacks, such as, the donor site morbidity, primitive injuries and high cost. The main disadvantage associated with the allograft is the dearth of osteogenic characteristics, harvesting and preservation [33]. The biomedical applications of synthetic materials are gaining importance since they display exceptional bioactivity, cellular responsiveness, entrapment and meticulous release of biologically active molecules. The tunable surface chemistry during synthesis opens up new horizons towards specific cellular targeted applications [34]. As a result HA finds applications in drug delivery [35], tooth enamel repairing [36], filler for bone repair [37], injectable bone cement [38], and as a coating for prosthetic implantation [39]. When HA is mixed with other phosphates, such as β -tricalcium phosphate (β -TCP) and calcium pyrophosphate, it promotes cell adhesion and osteogenic characteristics [40]. Bone has a very complex, hierarchical [41], 3D structure consisting of self-assembled discrete building blocks [42, 43]. HA can be synthesized by using various laboratory techniques, such as; wet chemical precipitation [44], sol-gel processing [45], solid-state reactions [46], chemical vapor deposition [47] and reverse micro emulsion [48]. Sol-gel process is well known for its flexibility and it offers different chemistries to fabricate a wide range of structural materials with particle sizes ranging from nano to micron scale [45, 49]. Many research groups have studied the effects of different parameters such as the reaction temperature and aging time on the morphology and crystallinity of synthetic calcium phosphate powders. Increase in overall crystallinity and crystal size were observed with temperature and ripening time. Increase in crystallinity was reported to produce particles with regular and smooth surfaces [50]. As expected, a change in Ca/P mole ratio strongly influenced the product formed where a decrease in Ca/P ratio resulted in an increase in the amount of β -TCP formed [51]. David et. al., studied

the effect of post synthesis thermal treatment on biphasic mixture of HA and β -TCP. They observed that after heat treatment at 900 °C, biphasic calcium phosphate particles were homogeneous in mixture and their micro-pore density was much more improved [52]. Post synthesis calcination also increased the particle size and crystallinity [53]. Similarly, Jung et. al., synthesized HA through spray pyrolysis by using high temperature flame and examined the effect of solvent on physical properties of the particles obtained. They used water and ethanol/water mixture (1/2 v/v) and observed that larger size and bimodal particles were formed through water only, while monodisperse and nano-sized particles were produced when ethanol/water mixture was used [54]. Due to considerable osteogenic properties of hydroxyapatite [55] and to improve the properties of composites [28, 30, 56], it has been incorporated into different natural and synthetic polymeric systems like chitosan [57], collagen [58], gelatin [59], polycaprolactone [60] and poly(lactic acid) [61].

1.3.2 Chitosan

Chitosan (CS) is a naturally occurring polysaccharide obtained by the deacetylation of chitin. Chitosan, which is more soluble in aqueous and organic solvents than chitin, has potentially collagen replacing characteristics. Due to the non-toxic degradation products and its biocompatibility, chitosan has become inevitable source for future research and development in the field of tissue engineering and regenerative medicine applications [62, 63]. A critical requirement for scaffolds used in hard tissue regeneration is their load bearing capability [64]. Chitosan being a flexible polymer does not meet the mechanical properties of natural bones so chitosan scaffolds cannot emulate all the requirements especially for hard tissue engineering applications. But these hurdles can be overcome by developing composite scaffolds. CS is semi crystalline polymer and its crystallinity depends on the degree of deacetylation. At 0% deacetylation (chitin) and 100% deacetylation, the crystallinity is maximum. CS is usually insoluble above pH-7 in aqueous solutions. However, due to the protonation of amino groups in acidic solutions below pH-5, it becomes soluble. CS degradation is also dependent on the degree of deacetylation. Lower levels of deacetylation causes rapid degradation and vice versa. When derivatives are attached to the side chains, packing density is lowered and degradation rate goes up.

This modification through derivatives can also be used to induce strength and promote biological activities [65].

1.3.3 Silk fibroin

Silk is a well-known natural polymeric material, which has been used in textile applications as fibers for centuries, due to its luster, durability and excellent mechanical properties [66, 67]. Silk and silk fibroin, a natural high molecular weight protein, also display excellent biocompatibility [68], biodegradability and mechanical strength. As a result silk finds considerable use in biomedical applications, as surgical sutures, carriers for therapeutic agent delivery and scaffolds in tissue engineering [67, 69-73]. The members of *Arcachnida* class and many worms of *Lepidoptera* produce silk. The diameter of *Bombyx mori* (*B. mori*) silk cocoons cover a wide range from 0.25 to 10 μm . *B. mori* silkworm silk fibroins consist of two proteins, light chain (~ 26 kDa molecular weight) and heavy chain (~ 390 kDa), at a ratio of 1:1, linked together through a disulfide bond [74]. Silk fibers are also coated with a hydrophilic protein, sericin with a molecular weight in the range of 20-310 kDa. Sericin coating makes up about 25-30 weight% of *B. mori* cocoons and needs to be removed through degumming process to get individual silk fibroin fibers [69, 75]. As shown in **Error! Reference source not found.**, primary structure of heavy chain of *B. mori* silk fibroin mainly consists of three different amino acids; glycine (Gly) (43-46 weight %), alanine (Aly) (25-30 weight %) and serine (Ser) (12 weight %). These amino acids are followed by tyrosine (5%), valine (2%) and others in much smaller amounts [73].

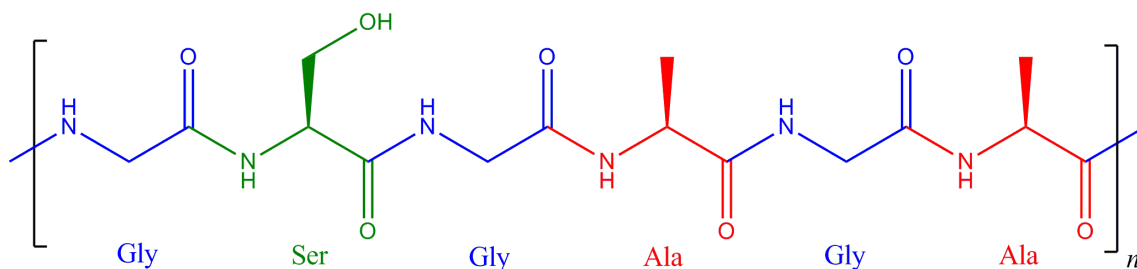


Figure 1.2. Primary structure of the heavy chain of *Bombyx mori* silk fibroin

In silk fibroin, low molecular weight or light chains consist of about 47 weight% residues of hydrophobic amino acids and they cannot form crystalline structures. On the other hand heavy chains, which serve as the main structural component of silk fibroin, form discrete β -sheet crystallites and provide the mechanical strength and integrity to the system [76, 77].

As a natural protein, silk displays typical structural and morphological features, such as crystalline and amorphous regions observed in all proteins. Crystalline regions in silk fibroin consists of α -helical (silk-I), β -folded (silk-II) and much less studied hexagonal type crystalline (silk-III) structures [78]. *B. mori* silk fibroin is comprised of $13\pm 5\%$ Silk-I, which is water soluble and $56\pm 5\%$ silk-II that is water insoluble. Crystalline regions contain about 60-70% of total fibroin and remaining amorphous region displays disordered conformation in form of random globules [79]. Silk-I can be converted into Silk-II by methanol, hydration, spinning or heat treatment [69].

Silk cocoons are degummed and sericin is removed in alkaline solutions. The structure and concentration of alkaline reagent, processing time and temperature strongly influence the morphologies and mechanical properties of the degummed fibers. Utilization of harsh chemicals and reaction conditions and long and repeated treatment processes may cause serious degradation (hydrolysis) of the silk fibroin and result in lowering the molecular weight [80-82]. A large number of methods, such as treating with borax-HCl buffer, urea, enzymes and most commonly use of 0.02M solution of sodium carbonate and bicarbonate have been reported in the literature for degumming of the cocoons to get clean silk fibers [62, 83]. Degumming conditions not only affect the mechanical properties but also the cyto-compatibility of silk fibroin [77]. After degumming silk fibers are usually dissolved in 9.3 M LiBr solution [70, 72, 84-86], $\text{CaCl}_2/\text{C}_2\text{H}_5\text{OH}/\text{H}_2\text{O}$ (1:2:8) solution [87-90] or other salt solutions [91, 92]. The main drawback of all these methods is that, since all solutions contain inorganic salts, at the end of the dissolution process it is necessary to apply dialysis to remove the salts in order to obtain pure silk fibroin [93]. In dialysis process, porous membranes with specific molecular weight cut off are used to remove the impurities from silk solution. After long process of dialysis, silk fibroin solution in water is obtained and can be concentrated if needed. However, even after purification, aqueous silk fibroin solution may still contain salt impurities. Silk fibroin can be processed into non-woven mats [94, 95], films [96, 97], freeze-dried sponges [98-100], hydrogels [70,

101], or can be blended with other polymers to prepare composites for specific targeted applications [100, 102-104]. Silk fibroin is extensively used in tissue engineering applications, including bone [105], cardiac [106], nerve tissue engineering [107] and drug delivery applications [108]. Endothelial cells [96], mesenchymal stem cells [99], nerve cells [109], cardiac cells [110], fibroblast cells [84, 94] and osteoblast like cells [102] have been seeded onto silk fibroin based scaffolds and all were found to proliferate and grow well.

1.3.4 Poly(lactic acid)

PLA is a thermoplastic, aliphatic polyester derived from renewable resources such as corn starch and sugarcane. PLA is a biocompatible and biodegradable polymer. It can be degraded in the human body and biotic environments through the hydrolysis of its aliphatic ester linkages [111]. Owing to its biodegradability and biocompatibility PLA is also one of the few synthetic polymers approved by the FDA for biomedical applications [112]. D- and L- are two optical isomers of lactic acid and PLA can be synthesized through ring-opening polymerization of lactides. Due to FDA approval, PLA can be used as medical implants in different forms such as, orthopedic device, mesh, screws, pins and rods [113]. It is also being used as a polymeric carrier for drug release applications, template for cell growth and tissue scaffolding [114].

1.3.5 Polycaprolactone

Polycaprolactone (PCL) has considerable potential in biomedical applications due to its unique properties such as; high crystallinity, excellent biocompatibility, appropriate mechanical strength and considerable biodegradation properties [115]. In tissue regeneration, low bioactivity of PCL hinders its applications due to lack of bio-functionalized groups on the surface. These privations reduce cells affinity towards surface and due to lack of cellular interactions, tissue growth gets lower [3]. Poor hydrophilicity of PCL also causes poor cells adhesion, their migration, proliferation and differentiation [116]. However, this problem can be overcome through surface modifications [117-119] or incorporation of bioactive materials [120-122]. PCL is being used for various

biomedical applications and has widely been studied for cardiac [123], bone [124], nerve [125], skin [126-129] and drug delivery applications [130, 131]. PCL with different molecular weights can be synthesized through catalytic ring-opening polymerization (ROP) reaction. These reactions can be carried out in bulk or in solution. The reaction mechanisms involved can be cationic, coordination insertion or anionic. High molecular weight polyesters can only be obtained by using coordination insertion or anionic ring opening polymerization methods [132].

1.3.6 Polyurethane

Synthetic thermoplastic elastomers (TPE) are one of the most important classes of materials that find applications in many fields like textiles [133], protective coatings [134], sports [135], automotive [136], wound dressings [137] and tissue engineering applications [138, 139]. Conventionally ABA triblock copolymers, composed of hard (A) and soft blocks (B), are prepared through living anionic polymerization, controlled free radical polymerization and ring opening polymerization. Segmented $(AB)_n$ thermoplastic elastomers provide much wider range of applications than ABA triblock due to broader choice of materials. Properties depend on chemical structures and composition of hard and soft segments. Irrespective of their hard content structures, segmented, thermoplastic polyurethane (TPU) copolymers are conventionally synthesized by step-growth polymerization usually through pre-polymer method [140]. Generally, soft segments (SS) are hydroxyl or amine terminated aliphatic polyesters, polyethers, polyisobutylene, polycarbonates, and polydimethylsiloxane oligomers having glass transition (T_g) below the room temperature. Hard segments (HS) are generated by the reaction of diisocyanates with diol or diamine chain extenders, leading to urethane or urea groups respectively. Segmented TPUs can be schematically represented as shown in Figure 1.3. Strongly hydrogen-bonded urethane and/or urea HS groups form stiff microphase separated domains in TPUs since they are physically cross-linked and possess high T_g or T_m . As a simplification, SS can be considered as “elastomeric springs” whereas, HS can be assumed as “rigid rods” depending on their chemical structures and nature. Through proper selection and design of SS and HS segments and their ratios, it's possible to synthesize TPUs of desired strength, flexibility and various other bulk and surface properties [141,

142]. Segmented thermoplastic polyurethanes can also be synthesized without chain extenders. These polymers also display interesting characteristics and sometimes the properties may compare with chain extended polyurethanes and polyureas [140]. Poly(propylene glycol), poly(ethylene glycol) polycaprolactone and glycolic acid are commonly used as polyols in biodegradable synthesis of polyurethanes (TPUs) [143]. PCL is an interesting material with enormous applications including biodegradable and biocompatible scaffolding in tissue engineering [144] and it can be hydrolyzed, resulting in non-toxic by products and can be metabolized as well [143].

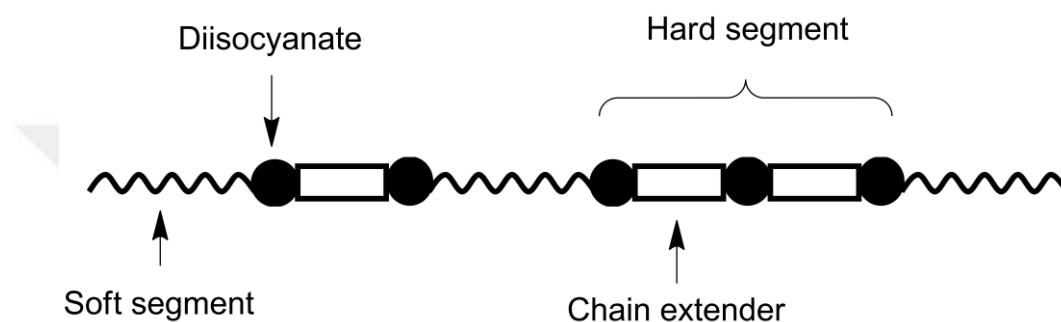


Figure 1.3. Schematic demonstration of segmented TPUs

In the hard segment of TPUs, aliphatic, aromatic, polycyclic or cycloaliphatic diisocyanates are used. Among these, aliphatic diisocyanates yield less rigid TPUs that show better oxidative properties [134]. For production of degradable TPUs, some commonly used diisocyanates are isophorone diisocyanate (IPDI), 1,4-butylene diisocyanate (BDI) and 1,6-hexamethylene diisocyanate (HDI). HDI is being used most commonly for synthesizing biomedical TPUs with relatively less toxic 1,6-hexamethylene diamine as by product [145, 146]. TPUs based on aromatic diisocyanates such as toluene diisocyanates (TDI) and 4,4'-methylenediphenyl diisocyanate (MDI) produce toxic byproducts [146].

1.3.7 Hybrid materials and composites

Bone has a very complex, 3D hierarchical structure, which is formed through the fairly intricate self-assembly of the individual building blocks [42, 43]. Close resemblance of the chemical structure, physical and chemical properties of hydroxyapatite with bones, makes it an important inorganic additive for the preparation of polymeric composites that can be

used as scaffolds for bone tissue engineering [147-150]. In general, polymers, metals and ceramics can all be used as scaffolds for tissue engineering applications. Each class of material has some advantages and disadvantages. Polymers offer ease of processing by various methods, good biocompatibility and degradation rate but may lack the desired mechanical properties [151]. Metals on the other hand have good mechanical properties but somewhat poor corrosion resistance. In general, ceramics may show good biocompatibility but they are fairly brittle and somewhat difficult to process [152, 153].

Considering all the properties required in scaffolds to be used in bone regeneration, including biocompatibility, cell proliferation and growth, biodegradability, mechanical integrity and flexible processing methods, the optimum system seems to be polymer matrix composites containing hydroxyapatite. In organic/inorganic composites, size and distribution of inorganic particles in the polymer matrix play critical role in bio-mineralization. For example, 20-40 nm sized HA nanoparticles are reported to be desirable for assisting the cellular functions [154].

In order to obtain optimum performance in the nanocomposites, a critical step is the preparation of stable dispersions of inorganic particles in polymeric solution. In bare form, ceramic nanoparticles have a great tendency to agglomerate so during composite solutions preparation, avoiding these agglomerations and sedimentation become critical and sometimes have to compromise on targeted properties. Agglomeration free dispersions have been obtained by applying high power sonication energy [155] and through surface modification of hydroxyapatite particles [156, 157].

This agglomeration and sedimentation can simply be overcome by choosing the proper solvent(s) because some solvents may provide good dispersion but not good long term stability due to clustering and sedimentation of HA [158].

In skin tissue engineering applications, desirable good mechanical properties and bioactivity are pre-requisite. For this reason, hybrid scaffolds can be developed from natural and synthetic biomaterials. Excellent biocompatibility, bioactivity and resemblance to natural ECM of natural materials can be combined with better mechanical properties of synthetic ones. This modified surface of synthetic biomaterials helps to attach and promote cell growth [159].

1.4 Fabrication techniques

1.4.1 Additive manufacturing techniques

A wide range of fabrication techniques have been developed for the preparation of scaffolds that can generate materials with different shapes, sizes, porosities, and architectures [1]. Three dimensional scaffolds can function as templates that provide temporary structural support to the cells and allow new tissue growth [160]. For this purpose, a high degree of porosity is required to facilitate the cell seeding, adhesion and diffusion through the structure for both cells and nutrients.

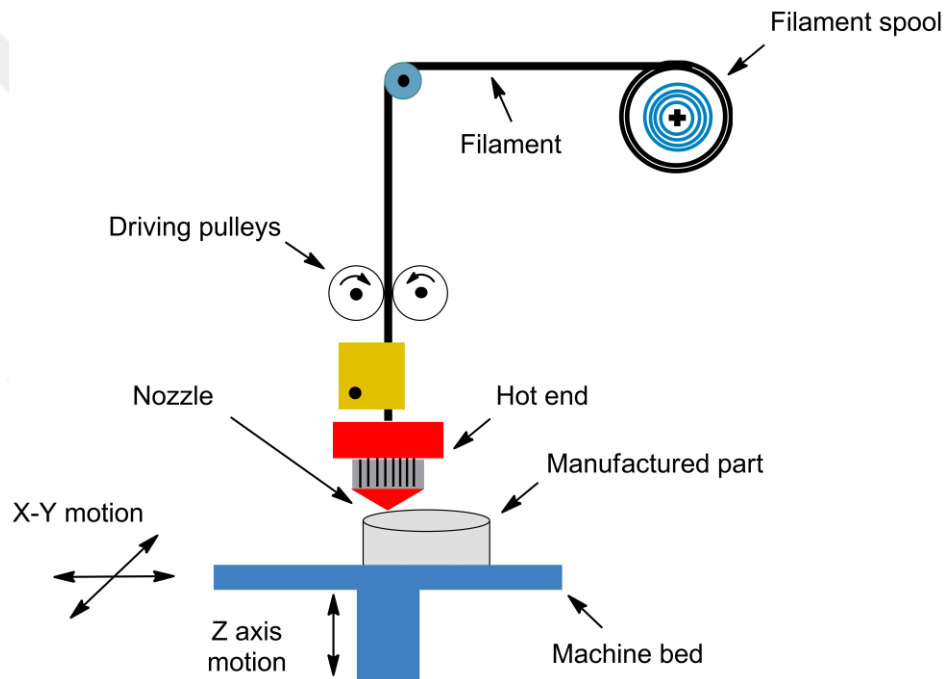


Figure 1.4. Schematic representation of basic fused deposition modelling process

To obtain 3D scaffolds some of the most widely used techniques include chemical [161] and physical [162] foaming, porogen leaching [163], emulsion templating [164], thermally induced phase separation [165, 166], and solid freeform fabrication (SFF) techniques [167, 168]. With solid freeform fabrication techniques, 3D scaffolds can be produced using computer aided designs. Compared to others, SFF techniques enable greater control of scaffold architecture and pore network. Various SFF techniques have been developed including selective laser sintering [169], stereo-lithography [170, 171], inkjet printing

[171], and fused deposition modelling (FDM) [172]. Fused deposition modelling offers great ease and flexibility in handling and processing of the materials. In this method, structures are created by layer by layer deposition of thermoplastic polymers using a 3D printer. First, thermoplastic filament is loaded into the printer. Then the polymer is softened above its melting point and extruded through the nozzle. Nozzle head moves along x and y directions according to the CAD design. After deposition of the first layer, build platform is lowered and next layer is built according to pre-set design. This process is repeated until 3D scaffold is achieved [173]. Basic FDM process is illustrated in Figure 1.4. PCL [174] and PLA [172, 175] are two of the most commonly used polymeric materials to fabricate 3D scaffolds through FDM technique.

1.4.2 Electrospinning technique

Electrospinning has emerged as a prevalent technique to fabricate fibrous scaffolds. These fibrous scaffolds consist of nano- to micrometer sized fibers that provide high surface area to volume ratio. These electro-spun fibrous scaffolds can mimic native ECM by incorporating suitable substrate and moieties. The functional environment provided by these nano-fibrous scaffolds can accommodate cells and helps them to grow and proliferate. Due to being a low-cost, simple and versatile technique, electrospinning has found its applications in various tissue engineering applications like bone, cartilage, skin, vascular and therapeutic agent's delivery as well [176]. Basic system of electrospinning consists of; (i) a polymer melt/solution pumping system, (ii) a high voltage power supply and (iii) a collecting substrate. The pumping system provides feed of solution/melt at a predefined constant rate. This solution is introduced in a syringe having a metallic needle. One end (+ve or -ve) of high voltage power supply is connected to the needle tip and the other to ground or opposite to needle. The electrostatic force generated in this way overcomes the surface tension in solution/melt droplet and eventually pulls out polymer solution/melt due to ground/opposite charge on the collector. The basic difference between electrospinning and conventional spinning methods is the tensile force responsible for originating fiber jet. In electrospinning, jet is initiated due to the force generated from potential difference between charged solution and grounded collector while in

conventional methods, tensile force is produced through mechanical means [177, 178]. Basic electrospinning setup is shown in Figure 1.5.

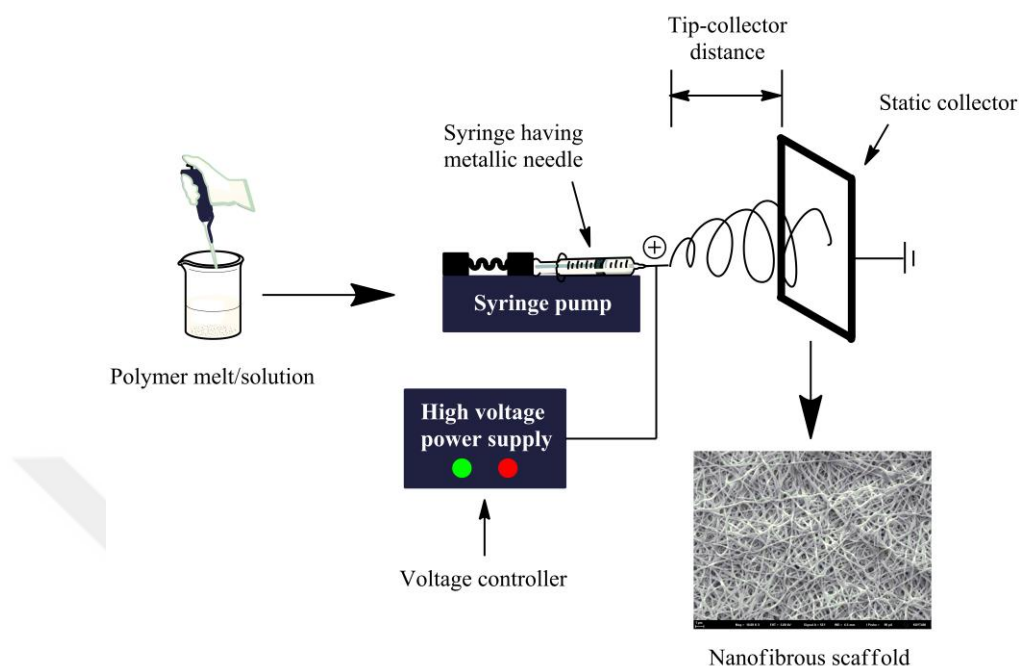


Figure 1.5. Basic electrospinning setup for nano-fibrous scaffolds development

There are various processing factors affecting morphology of the fibers obtained, such as; the static voltage, working (tip to collector) distance, solution viscosity and feed rate [179]. Nano-fibrous scaffolds developed through electrospinning are in shape of non-woven fibers embedded together in the form of a sheet. Earlier development of electrospun membranes mimicking the morphology of native ECM compelled researchers to focus on this technology to make more sophisticated scaffolds. Later on research resulted in development of controlled architectures of fibers in mesh like aligned, patterned surfaces and possessing gradient properties. Further modifications in basic electrospinning setup enabled researchers to make more advanced fibers like core-shell fibers and having complex geometries [180]. Fiber size and morphology also has a significant influence on cellular behavior. List of possible materials to be used for electrospinning is almost endless and numerous materials have been successfully converted to fibrous membranes and scaffolds. Ease of the electrospinning process further opened new horizons for researchers to make functional membranes by incorporation of other functional bioactive moieties like growth factors and proteins etc. [181].

CHAPTER 2

HYDROXYAPATITE SYNTHESIS¹

2.1 Summary

Synthesis of hydroxyapatite (HA) through sol-gel process in different solvent systems was investigated. Calcium nitrate tetrahydrate (CNTH) and diammonium hydrogen phosphate (DAHP) were used as calcium and phosphorous precursors, respectively. Three different synthesis reactions were carried out by changing the solvent media, while keeping all other process parameters constant. 0.5 M aqueous DAHP solution was used in all reactions while CNTH was dissolved in distilled water, tetrahydrofuran (THF) and N,N-dimethylformamide (DMF) at a concentration of 0.5 M. Ammonia solution (28-30%) was used to maintain the pH of the reaction mixtures in 10-12 range. All reactions were carried out at 40 ± 2 °C for 4 h. Upon completion of the reactions, products were filtered, washed, and calcined at 500 °C for 2 h. It was clearly demonstrated through various characterization techniques that, the dielectric constant and polarity of the solvent mixture strongly influences the chemical structure and morphological properties of calcium phosphate synthesized. Water based reaction medium, with highest dielectric constant, mainly produced β -calcium pyrophosphate (β -CPF) with a minor amount of HA. DMF/water system yielded HA as the major phase with a very minor amount of β -CPF. THF/water solvent system with the lowest dielectric constant, resulted in the formation of pure HA.

¹ This work has been published as, “Nazeer, M. A., Yilgor, E., Yagci, M. B., Unal, U., & Yilgor, I. (2017). Effect of reaction solvent on hydroxyapatite synthesis in sol–gel process. *Royal Society open science*, 4(12), 171098.”

2.2 Materials and methods

2.2.1 Materials

Reagent grade diammonium hydrogen phosphate ((NH₄)₂HPO₄), calcium nitrate tetrahydrate (Ca(NO₃)₂·4H₂O), tetrahydrofuran (THF), N,N-dimethylformamide (DMF) and ammonia solution (28-30%) (NH₄OH) were received from Merck and used as received without further purification. Deionized and triple distilled water was used for all experiments. Dielectric constants (ϵ) of THF, DMF and water are 7.58, 36.7 and 80.1 respectively [182-184].

2.2.2 Methods

2.2.2.1 Hydroxyapatite synthesis

Calcium phosphate synthesis was carried out as reported earlier, [185, 186] with minor variations. Three different synthetic procedures were followed as presented in Figure 2.1. DAHP was dissolved in distilled water in all reaction schemes, while CNTH was dissolved in distilled water (Scheme-1), THF (Scheme-2) and DMF (Scheme-3). All other reaction parameters such as temperature, time, addition rate, washing cycles, drying time and temperature were kept the same.

The pH of water based solutions was adjusted in the range of 10-12 by dropwise addition of NH₄OH solution, whereas for organic solvents, it was adjusted in 5 mL of water and then diluted with organic solvent. CNTH (100 mmole) was dissolved in water, THF and DMF while DAHP (60 mmole) was dissolved in water to reach the final concentration of 0.5 M each. CNTH solution was introduced into a 500 mL three-neck round bottom Pyrex flask, equipped with an overhead stirrer, addition funnel and thermometer. DAHP solution was added with an addition rate of ~40 drops/min through an addition funnel while the mixture was vigorously stirred at 250 rpm. Water bath was used to maintain the temperature at 40±2 °C. After complete addition of DAHP solution,

reaction was continued for 4 h. After the completion of the reaction product obtained was filtered through a filter paper (Filter-lab REF. 1525), washed several times with distilled water and then dried in oven at 60 °C for 24 h. All powders obtained were calcined in a furnace at 500 °C for 2 h, which was heated with a heating rate of 10 °C/min. After calcination, the powders were directly used for characterization.

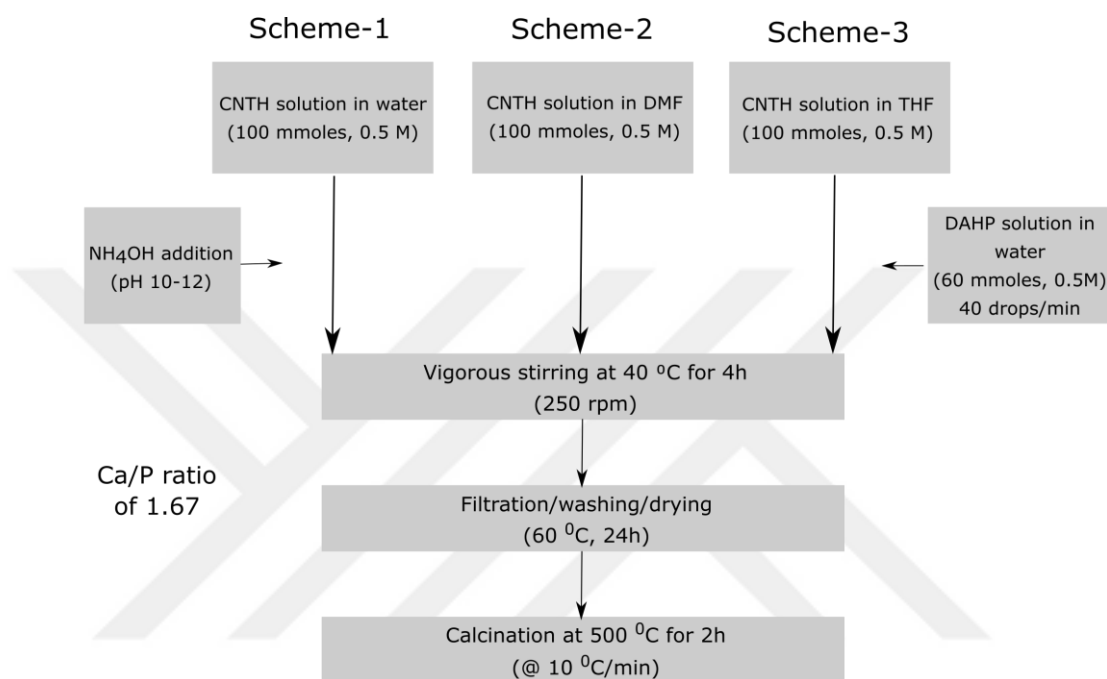


Figure 2.1. Schematic representation of the reaction schemes followed in calcium phosphate synthesis through sol-gel process

2.2.2.2 Characterization techniques

ATR-FTIR spectra were acquired on a Thermo-scientific iS50 FTIR with ATR attachment. Absorption spectra were collected by attaining 64 scans at auto-gain with a resolution of 2 cm^{-1} . X-Ray Diffraction (XRD) diffractograms were obtained using a Bruker D8 Discovery X-Ray Diffraction system. Cu $K\alpha$ X-ray generator with a wavelength of 1.54184 Å at a voltage 40 kV and 40 mA current was used. Surface and morphological analyses were done on Zeiss Ultra Plus Field Emission Scanning Electron Microscope (FESEM) equipped with energy dispersive X-ray detector for elemental analysis. Powder sample was dispersed at 0.1% (w/w) in formic acid and spin coated on silicon wafer to make surface fairly conductive without using gold/carbon sputtering. Secondary electron

detector images were obtained to analyze the morphology of the nanoparticles produced. Raman analysis was performed on a Renishaw Invia-Raman microscope by using either 532 or 785 nm laser sources. Ca/P atomic ratio was determined by X-ray fluorescence microscopy (XRF) using Bruker Tiger S8 instrument.

2.3 Results and discussion

2.3.1 X-ray diffraction analysis

Crystallographic structures of the calcium phosphate synthesized in different solvents were determined by wide angle x-ray diffraction analysis (WAXD). Diffractograms for different samples are reproduced in Figure 2.2, where characteristics peaks for HA are shown with red arrows and peaks for β -calcium pyrophosphate (β -CPF) are marked with black arrows at their corresponding 2θ angles.

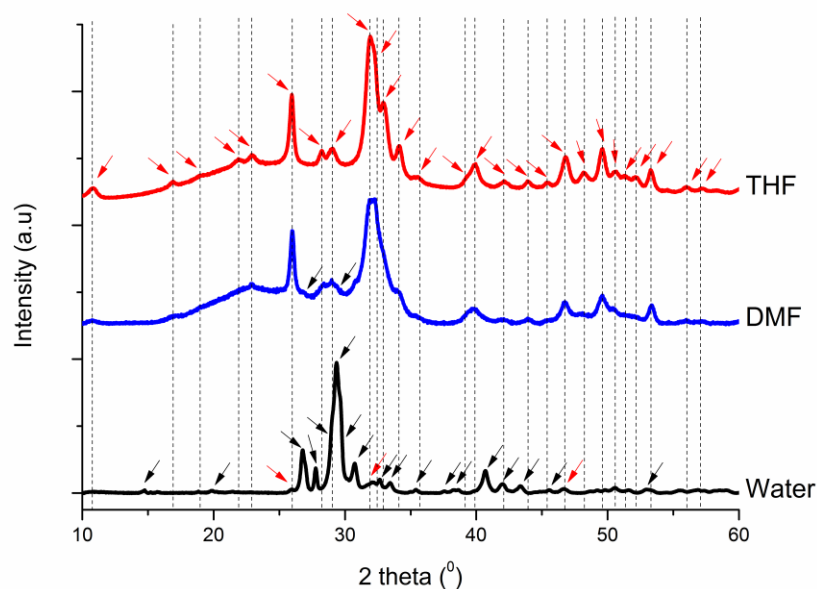


Figure 2.2. XRD analysis of calcined calcium phosphate powders synthesized by using water, THF and DMF as the reaction co-solvent.

Table 2.1. XRD analysis of Hydroxyapatite and β -Calcium pyrophosphate

Hydroxyapatite $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$			β -Calcium pyrophosphate $\beta\text{-Ca}_2\text{P}_2\text{O}_7$		
Angle 2θ (degrees)	d value ($\text{\AA}$)	h, k, l	Angle 2θ (degrees)	d value ($\text{\AA}$)	h, k, l
10.85	8.15	1,0,0	14.68	6.03	0,0,4
16.89	5.25	1,0,1	19.80	4.48	1,0,4
18.84	4.71	1,1,0	26.91	3.31	2,0,1
21.79	4.08	2,0,0	27.68	3.22	2,0,2
25.98	3.43	0,0,2	28.87	3.09	2,0,3
28.23	3.16	1,0,2	29.56	3.02	0,0,8
28.96	3.08	2,1,0	29.87	2.99	2,1,0
31.82	2.81	2,1,1	30.78	2.90	2,1,2
32.29	2.77	1,1,2	32.55	2.75	2,0,5
32.94	2.72	3,0,0	33.41	2.68	2,1,4
34.16	2.62	2,0,2	35.27	2.54	2,1,5
35.52	2.53	3,0,1	37.41	2.40	2,1,6
39.29	2.29	1,2,2	38.52	2.34	1,1,9
39.84	2.26	1,3,0	40.61	2.22	3,0,1
42.05	2.15	1,3,1	42.01	2.15	3,0,3
44.03	2.06	1,1,3	43.19	2.09	3,0,4
45.48	1.99	2,0,3	45.47	1.99	1,1,11
46.80	1.94	2,2,2	52.78	1.62	4,1,1
50.55	1.80	3,2,1			
51.33	1.78	1,4,0			
52.18	1.75	4,0,2			
53.44	1.71	0,0,4			
55.98	1.64	3,2,2			
57.29	1.61	1,3,3			

ATR-FTIR analysis XRD diffractograms for all samples were analyzed through DIFFRACT.SUITE EVA V.4.2. Samples were compared with database, matched with cards for HA (PDF-04-014-8416) and β -CPF (PDF-00-009-0346) and their respective diffractograms are provided as Appendix-I. Diffraction peaks for HA and β -CPF crystal planes along with h, k, l indices, d spacing value and their respective angles are tabulated in Table 2.1.

2.3.2 ATR-FTIR Analysis

To identify the characteristic functional groups in the powders synthesized, FTIR spectroscopy was utilized. The spectra obtained are provided in Figure 2.3, where hydrogen-bonded OH stretching band appears at 3570 cm^{-1} , which is more obvious in THF and DMF based products due to the presence of HA [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$] and less observable in water based product, which is mainly β -CPF [$\beta\text{-Ca}_2\text{P}_2\text{O}_7$].

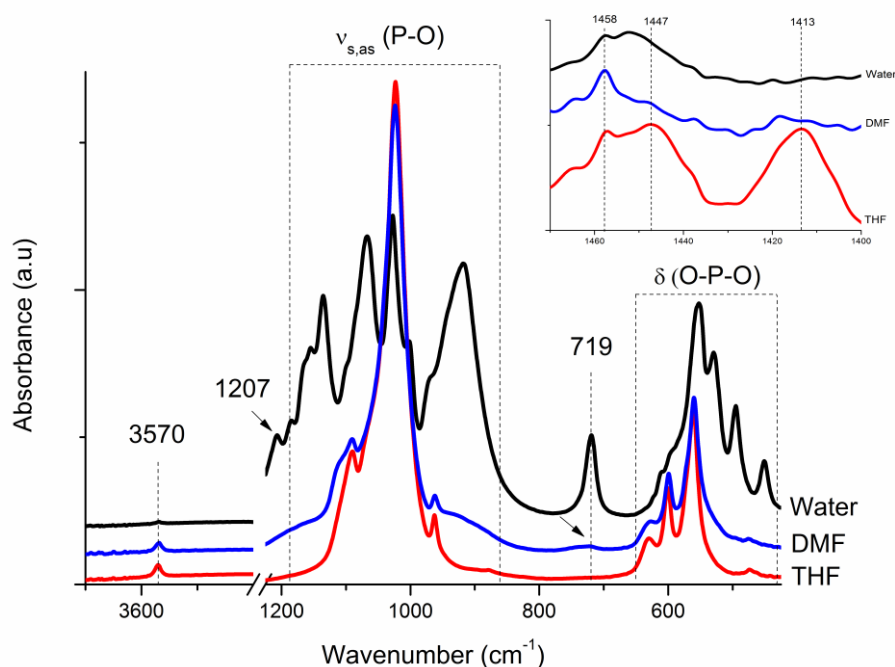


Figure 2.3. ATR-FTIR analysis for synthesized calcium phosphate powders obtained through different reaction schemes

Symmetrical and asymmetrical PO_4^{3-} stretching modes characterized by complex bands in the region of $1200\text{-}900\text{ cm}^{-1}$ are observed, which are typically associated with hydroxyapatite. Bending vibrations for O-P-O appear in the range of $650\text{-}425\text{ cm}^{-1}$. There are two more absorption bands appearing at 1207 and 719 cm^{-1} , that correspond to pyrophosphate ($\text{P}_2\text{O}_7^{4-}$) stretching modes [185]. These bands are observed significantly only in water based synthesis due to the large presence of β -CPF phase. Very weak signal at 719 cm^{-1} as indicated by black arrow head was present in DMF based synthesis. Additionally, some minor and weak bands were also observed in the range of $1470\text{-}1400\text{ cm}^{-1}$ enlarged and represented in the inset graph of Figure 2.3. These bands appearing at 1413 , 1447 and 1458 cm^{-1} , can be assigned to CO_3^{2-} [187, 188]. This is probably due to the absorption of carbon dioxide from atmosphere, which interacts with nano-HA powders. THF based system shows more carbonate presence than others because hydroxides have very high affinity to carbonate, especially at high pH values. This suggests that some tracing amounts of PO_4^{3-} have been substituted by carbonate [55]. Ambient CO_2 dissolved can be limited by either performing reaction under lower ambient conditions or sintering synthesized powder at elevated temperature. Replacement of CO_3^{2-} with PO_4^{3-} enhances hydroxyapatite dissolution and results in the reduction of the crystallite size [189].

2.3.3 Raman analysis

Due to the different bonding structures present in various calcium phosphates, we used Raman technique to distinguish them from each other. Raman spot analysis and Raman mapping were done for different powder samples obtained through different reaction schemes and results are represented in Figure 2.4 and Figure 2.5, respectively. Raman spectra for powders synthesized in THF and water were obtained by using solid state laser of 532 nm wavelength having beam size of $1.5\text{-}2\text{ }\mu\text{m}$ in diameter, while for DMF based synthesis analysis was done with line laser (785 nm) of dimensions $1.5\text{-}2\times 20\text{ }\mu\text{m}$. As DMF based system has more mixed phases, we accordingly analyzed it through line laser to get signals from minor phase of β -CPF as well.

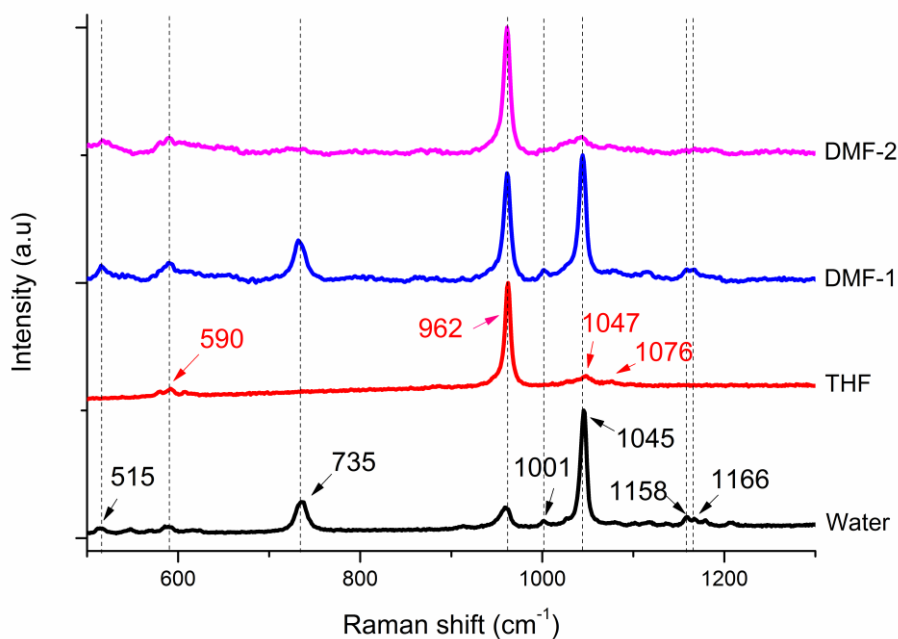


Figure 2.4. Raman analysis of calcium phosphate powders obtained from different reaction schemes

In DMF based synthesis, analysis was done at two different points that yielded different spectra indicating the presence of a minor β -CPF phase in a mixture with bulk HA phase. Raman peaks for β -CPF appear at 515, 735, 1001, 1158, 1166 and the most dominating peak at 1045 cm⁻¹ as indicated by black arrow heads in Figure 2.4, while for HA, peaks appear at 590, 1047, 1076 and the major peak at 962 cm⁻¹ [32, 190]. In DMF-2 spectrum, only peaks for HA phase were observed whereas for another spot analysis DMF-1, peaks for both HA and β -CPF were observed. These results confirm that, THF based synthesis resulted in only HA phase formation, water based synthesis yielded larger phase of β -CPF and a minor phase of HA while DMF based synthesis generated larger phase of HA and much minor phase of β -CPF. Raman mapping was also performed to show the presence of different phases in the samples. Laser of 532 nm was used and the results are reproduced in Figure 2.5. Red color shows the presence of HA and green color represents β -CPF phase present in calcium phosphate mixture. In water based synthesis, we can clearly see the mixture of HA and β -CPF, in DMF based synthesis, a fairly minor phase of β -CPF is

present as indicated by black arrows while THF based synthesis consists of purely HA phase.



Figure 2.5. Raman mapping of synthesized calcium phosphate phases (scale bar=20 μm), HA (red), β -CPF (green)

Raman spot analysis and mapping is a powerful tool for checking the purity of the materials synthesized by technically selecting appropriate irradiated laser type and analysis parameters. Even if material is in the powder form and it is “XRD amorphous” (i. e. does not produce distinct XRD peaks), it can still have strong peaks in Raman spectrum because Raman scattering is sensitive to ordering within atomic clusters, while X-ray diffraction is at larger scale [32]. These results strongly confirm our hypothesis that phase of synthesized calcium phosphate depends on the nature and properties of the solvent media, when other processing conditions (such as temperature, time) are kept constant.

2.3.4 Surface analysis

Secondary electron (SE) images of the synthesized calcium phosphate powders were collected on FESEM. All three powders were dispersed in formic acid at 0.1 w/w% and spin coated at 1000 rpm for 70 seconds on cleaned silicon wafers. After completely evaporating the solvent in a vacuum oven, samples were analyzed at accelerating voltage of 2 kV without gold/carbon sputtering. Acquired SE images are provided in Figure 2.6.

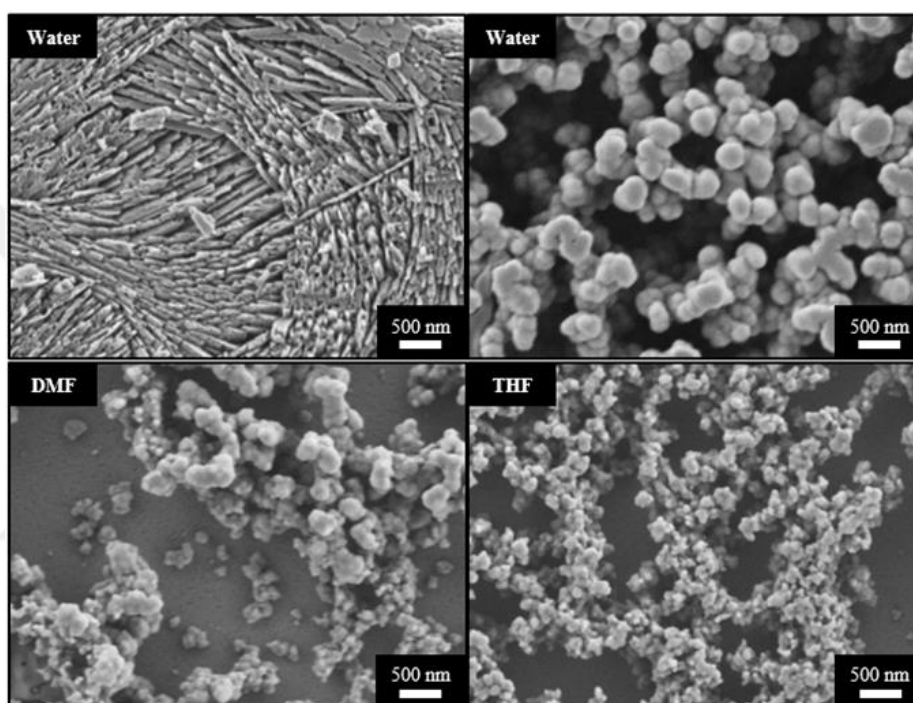


Figure 2.6. FESEM analysis of calcium phosphate powders synthesized in different solvent media

In water based synthesis, formation of biphasic calcium phosphate showing different morphologies were observed, where plate like structure corresponds to β -CPF. HA was observed as larger, spherical particles with fairly uniform sizes when compared to other samples. Higher amount of β -CPF was found to be present in the water based sample as compared to HA. In DMF based synthesis, only spherical particles were observed in the SE image analysis corresponding to HA particles because of very low quantity of β -CPF formed. Broader particle size distribution was also observed. In THF based synthesis spherical HA particles with fairly homogenous size distribution were observed. Water

based synthesis yields large amounts of β -CPF and minor amount of HA, whereas the DMF based product comprises of mainly HA with a small amount of β -CPF, while the THF based product purely consists of HA as discussed in FTIR, XRD and Raman sections.

2.3.5 *Effect of solvent on physical and chemical properties*

As we have demonstrated, the dielectric constant and polarity of the reaction solvent has a critical effect on the synthesis of calcium phosphate through sol-gel process. Solvent can influence the reaction rate and hydrolysis thus ultimately the structure of the synthesized material [191, 192]. Dielectric constant of a solvent, which is related to the strength of its intermolecular forces and polarity, determines the efficiency of the solvent in separating the electrolytes into ions. Solvents with high dielectric constants favor complete dissociation of electrolytes, while solvents with low dielectric constant cause ion pairing. Dielectric constant of solvent mixture can be calculated as a simple additive function of weighted average of the dielectric constants of mixture components [193, 194]. Calculated dielectric constant values for all three reaction schemes are given in Table 2.2. As Ca/P ratio for β -CPF and HA is 1 and 1.67 respectively, keeping all other parameters constant, we believe as the polarity of the reaction medium increases, reaction rate also increases, resulting in the formation of higher amounts of β -CPF rather than HA. Hence, concentration of individual phase in a multiphasic calcium phosphate synthesis can be monitored by carefully controlling the polarity and the dielectric constant of the solvent mixture. Surface area (S_{BET}) and average particle sizes were determined by N_2 adsorption isotherms according to BET (Brunauer-Emmett-Teller) procedure and are provided in Table 2.2. Results obtained are in good agreement with FESEM analysis. Water based synthesized powder has large plate like structure for β -CPF as compared to smaller spherical shaped HA particles in the other two solvents systems. Average particle size is very high as compared to others and hence very low surface area. Average particle size in THF based synthesis is slightly larger than DMF based system due to low rate of reaction [195]. Results obtained in THF and DMF based synthesis are almost comparable. For elemental analysis to compute Ca/P ratio, XRF analysis was performed and calculated Ca/P values are provided in Table 2.2. In water based synthesis, ratio is quite low, 1.07, because of the presence of larger β -CPF phase with a Ca/P molar ratio of 1.

Table 2.2. Influence of the dielectric constant of the solvent mixture on BET, XRF and XRD analysis of calcium phosphate synthesized (a=k value of 0.89, b=k value of 1)

		Water based synthesis	DMF based synthesis	THF based synthesis
Dielectric constant of solvent mixture		78.54	54.28	37.4
BET analysis	Average particle size, d_{BET} (nm)	247	73	80
	Surface area, S_{BET} (m^2g^{-1})	24	82	75
XRF analysis	Ca/P ratio	1.07	1.45	1.78
XRD analysis	Crystallite size (nm)	19.4 ^a	27.0 ^b	25.2 ^b
	Crystallinity (%)	70.7	25.9	31.9

DMF based synthesis has a much higher Ca/P ratio of 1.45, where HA is the major and β -CPF is the minor product. THF based synthesis yielded the maximum value of Ca/P ratio of 1.78, clearly indicating the formation of pure HA. Slightly higher Ca/P value of this system compared to the theoretical Ca/P value of 1.67 for pure HA phase can be due to replacement of phosphate groups with carbonates, as discussed in ATR-FTIR section. Broadening of the x-ray reflection peaks can be utilized to estimate the crystallite size in a direction vertical to the crystallographic phase by using Debye Scherrer Equation 2.1 [196],

$$D = \frac{k\lambda}{\beta_{1/2}\cos\theta} \quad \text{Equation 2.1}$$

where, D is the size in Å, k the shape factor and its value was used as 1 for THF and DMF based synthesized powder and 0.89 for water based powder due to the morphology difference. λ is the wavelength used, which is 1.54184 Å, θ is the diffraction angle and $\beta_{1/2}$ is the selected diffraction peak width at half height expressed in radians. (002) and (201) peaks were selected for HA and β -CPF crystal size calculation because of their prominent and distinct nature. Crystallite size and percent crystallinity for each synthesized powder were calculated/estimated using Diffract Eva[®] software and results are given in Table 2.2. Relatively smaller crystallite size and highest amount of crystallinity was observed in

water based calcium phosphate powder due to the presence of a fairly large β -calcium pyrophosphate phase in mixture. The reaction rate was highest among all reaction schemes due to the highest polarity and dielectric constant of the system that ultimately led to calcium pyrophosphate crystal growth instead of hydroxyapatite.

2.4 Conclusions

In this part of the study, critical influence of the dielectric constant and polarity of the reaction solvent on calcium phosphate synthesis was explored. Main aim of the study was the investigation of the solvent effect on the synthesis of hydroxyapatite with a Ca/P ratio of 1.67 through a sol-gel process. This was achieved by reacting “Ca” and “P” precursors in different solvent mixtures, where pure water, water/DMF and water/THF mixtures with different polarities and dielectric constants were used as the reaction mixtures. After washing, drying and calcination of the synthesized powder, physical and chemical characterization was done via FESEM, BET, ATR-FTIR, Raman, XRF and XRD techniques. Water based reaction medium with highest dielectric constant, yielded mainly β -calcium pyrophosphate with a minor amount of HA. In DMF/water based synthesis, HA was obtained as the major phase with a very minor amount of β -CPF. In THF/water solvent system with the lowest dielectric constant, pure HA was synthesized. Our results clearly show the significant effect of the dielectric constant and polarity of the reaction medium on calcium phosphate synthesis through sol-gel process, while keeping the other factors constant. By carefully controlling the solvent parameters, one can control the amount of different calcium phosphate phases produced, which may play critical roles for specific cellular activities and targeted applications.

CHAPTER 3

INTERCALATED CHITOSAN/HYDROXYAPATITE NANO-COMPOSITES²

3.1 Summary

Preparation and characterization of chitosan/hydroxyapatite (CS/HA) nanocomposites displaying an intercalated structure is reported. As discussed earlier HA was synthesized through a sol-gel process. Formic acid was introduced as a new solvent to obtain stable dispersions of nano-sized HA particles in polymer solution. CS/HA dispersions with HA contents of 5, 10 and 20% by weight were prepared. Self-assembling of HA nanoparticles during the drying of the solvent cast films led to the formation of homogeneous CS/HA nanocomposites. Composite films were analyzed by SEM, AFM, EDX, FTIR, XRD and TGA. SEM and AFM confirmed the presence of uniformly distributed HA nanoparticles on the chitosan matrix surface. XRD patterns and cross-sectional SEM images showed the formation of layered nanocomposites. Complete degradation of chitosan matrix in TGA experiments, led to the formation of nano-porous 3D scaffolds containing hydroxyapatite, β -tricalcium phosphate and calcium pyrophosphate. CS/HA composites can be considered as promising materials for bone tissue engineering applications

² This work has been published as, “Nazeer, M. A., Yilgör, E., & Yilgör, I. (2017). Intercalated chitosan/hydroxyapatite nanocomposites: promising materials for bone tissue engineering applications. Carbohydrate polymers, 175, 38-46.”

3.2 Materials and methods

3.2.1 Materials

Low molecular weight chitosan with a deacetylation degree of 75-85% was obtained from Aldrich. Formic acid (98%), ethanol, diammonium hydrogen phosphate ($(\text{NH}_4)_2\text{HPO}_4$) (DAHP), ammonia solution (28-30%) and calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$) (CNTH) were received from Merck and were used as received. Deionized, triple distilled water was used in all experiments. Nano-phasic hydroxyapatite (HA) was synthesized in our laboratories.

3.2.2 Methods

3.2.3 Hydroxyapatite synthesis

Hydroxyapatite was synthesized according to previous method explained in section 2.2.2.1 of Chapter 2 with minor modifications. In this synthesis, CNTH was dissolved in water/ethanol (1/12) mixture while DAHP was dissolved in distilled water and reaction was proceeded as before to yield hydroxyapatite. Subsequently, synthesized HA was calcined by heating to 500 and 1100 °C with a heating rate of 10 °C/min and keeping at respective temperature for an hour.

3.2.4 Chitosan/hydroxyapatite (CS/HA) nanocomposites development

2% w/w suspensions with varying concentrations of CS and HA were prepared in formic acid. HA heat treated at 500 °C was used for the preparation of CS/HA dispersions. HA suspensions in formic acid were prepared by magnetic stirring (60 min) followed by sonication (30 min). Separately, the required quantity of CS was dissolved in formic acid by using magnetic stirring. HA suspensions were filtered through 0.45 µm Teflon® membrane before mixing with the CS solution in a beaker, under magnetic stirring.

Homogeneous CS/HA dispersions obtained were poured into Teflon® molds to prepare the nanocomposite films. Composites films were obtained by drying in a fume hood, at room temperature, overnight and then in a vacuum oven at 60 °C, until constant weight, prior to analysis.

3.2.5 Characterization Techniques

ATR-FTIR spectra of HA and CS/HA nanocomposite films were collected on a Thermo-Scientific iS50 FT-IR equipped with single and multiple reflection ATR. Absorbance spectra were collected at autogain, using 64 scans at a resolution of 2 cm⁻¹. X-Ray Diffraction (XRD) analysis of HA and nanocomposites was performed on Bruker D2 X-Ray Diffractometer. Samples were scanned for 10-90° 2θ and 10 sec per step for total steps of 3920. Cu tube was used as x-rays generator with a wavelength of 1.54184Å at 30kV and 10mA. Surface images and elemental analysis of the samples were obtained on Zeiss EVO LS 15 Scanning Electron Microscope (SEM) equipped with an Energy Dispersive X-ray (EDX) detector. Prior to surface and elemental analysis, the samples were coated with 10 nm layer of gold through sputtering. For topographical analysis, secondary electron images were obtained at 7 kV excitation voltage, 20 μA beam current and 10 pA I-probe. Elemental analysis was performed using the same parameters except I probe of 1 nA. Atomic force microscopic analysis was performed on Bruker Dimension Icon AFM. Phase and height images were obtained by scanning an area of 5x5 μm² using standard tapping mode and a Bruker TAP525A tip with a force constant of 325 N/m and resonance frequency of 575 kHz. TGA analysis was performed on TA Instruments TGA Q500. Samples were heated from room temperature to 1000 °C with a heating rate of 10 °C/min in air with a flow rate of 60 mL/min. Stress-strain profiles for chitosan and its composite films were obtained on Instron Model 4411 universal testing machine, with crosshead speed of 25 mm/min at room temperature. Dog-bone shaped samples of initial length 24 mm were punched out of films with a standard die (ASTM D1708).

3.3 Results and Discussion

3.3.1 Characterization of hydroxyapatite

FTIR spectra of hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) after 1 h of heat treatment at 500° C (HA-500) and 1 h at 1100 °C (HA-1100) are provided in Figure 3.1(a). FTIR spectrum of HA-500 clearly shows the characteristic absorption bands for PO_4^{3-} at 1089 and 1029 cm^{-1} (asymmetrical P-O stretching), 961 cm^{-1} (symmetrical P-O stretching) and 599 and 558 cm^{-1} (O-P-O bending). It was observed that after heat treatment at 1100 °C some of the phases were converted to tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$) (β -TCP). The bands observed at 940 and 1188 cm^{-1} can be attributed to the converted PO_4^{3-} phases. Similarly, presence of new pyrophosphate $\text{P}_2\text{O}_7^{4-}$ phases can also be observed at 725 cm^{-1} and 1213 cm^{-1} . These converted phases might be of great importance while selecting combinations of these materials for specific cell interactions in substrates as the studies show that incorporation of TCP and HA promotes cell proliferation, migration and their survival [197].

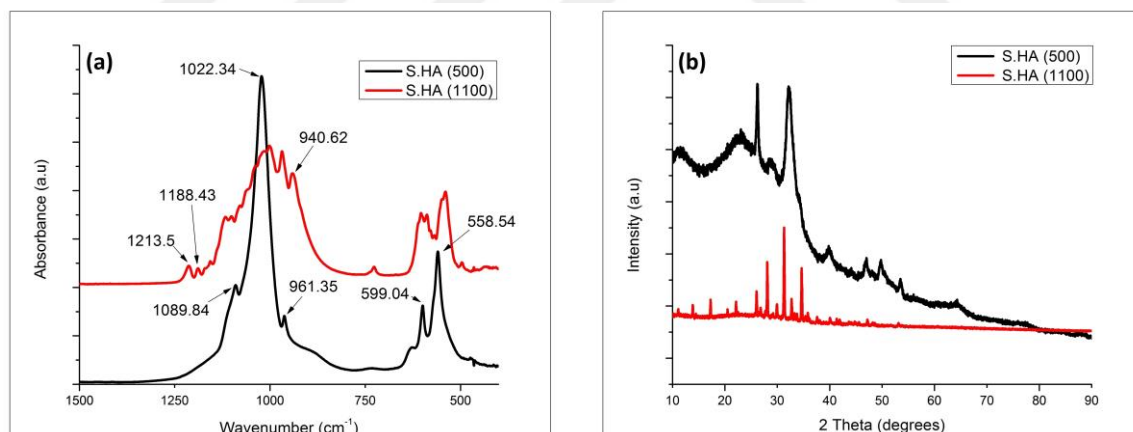


Figure 3.1. (a) ATR-FTIR spectra (b) XRD analysis of hydroxyapatite; after 1 h heat treatment at 500 °C (HA-500) and 1100 °C (HA-1100)

Crystallinity of HA before and after heat treatment was investigated by XRD analysis. Each sample was analyzed for ~11 hours. XRD patterns of HA-500 and HA-1100 are reproduced in Figure 3.1(b). Heat treatment at 1100 °C has induced further crystallinity in HA-1100, resulting in sharpening of the peaks and appearance of new phases due to the

conversion of HA ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) to β -tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$) and calcium pyrophosphate ($\text{Ca}_2\text{P}_2\text{O}_7$) at high temperatures, as discussed before in the ATR-FTIR analysis. XRD pattern of HA-500 synthesized in our laboratories matches well with standard PDF 01-080-3958 provided as Appendix-II.

3.3.2 Characterization of chitosan/hydroxyapatite (CS/HA) nanocomposites

3.3.2.1 ATR-FTIR and XRD analysis of CS/HA nanocomposites

Chemical structure, composition and crystallinity of CS/HA nanocomposites were investigated by ATR-FTIR spectroscopy and XRD. FTIR spectra of chitosan, hydroxyapatite and CS/HA films with different concentrations are reproduced in Figure 3.2(a).

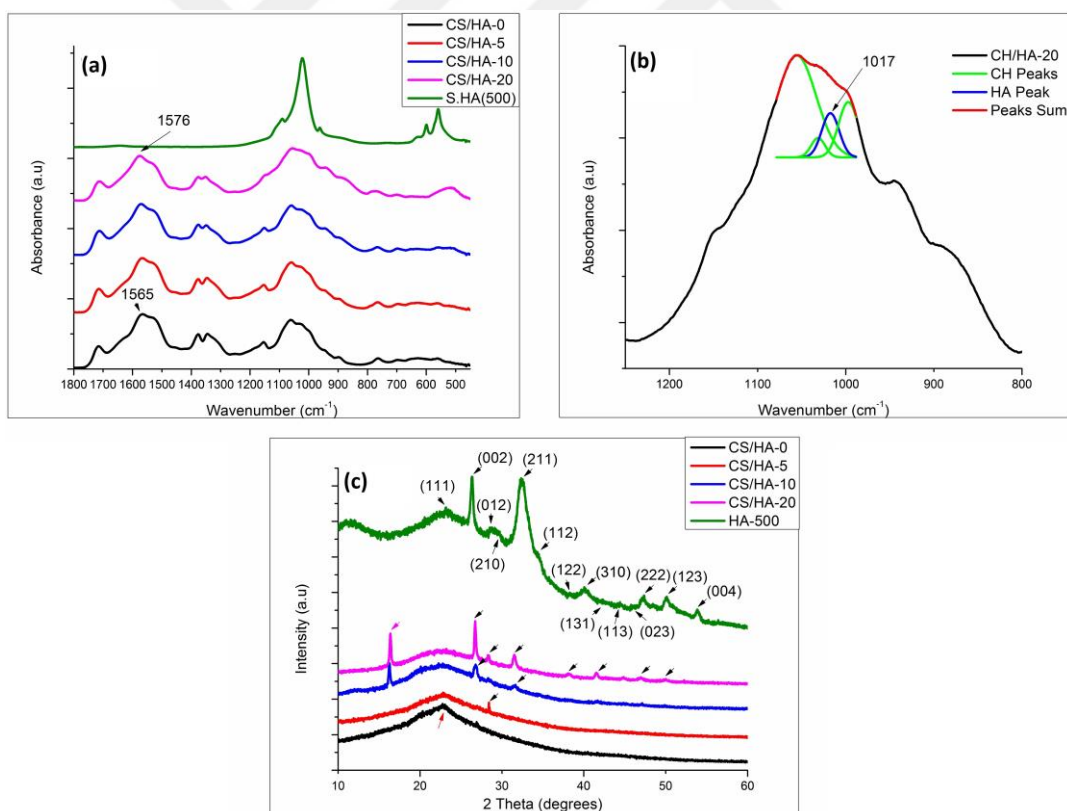


Figure 3.2. ATR-FTIR spectra of; (a) hydroxyapatite, chitosan and their composites; (b) de-convoluted spectrum of CS/HA-20; (c) XRD analysis of HA-500, chitosan and their composites

A slight shift in the N-H bending peak in chitosan from 1565 cm^{-1} to 1576 cm^{-1} has been observed in the composite films due to its interaction with HA nanoparticles through hydrogen bonding [198]. Unfortunately, since the characteristic IR absorption bands of hydroxyapatite in $1200\text{--}900\text{ cm}^{-1}$ region are overlapping with those of chitosan, it is fairly difficult to get quantitative information from these spectra. To obtain more reliable quantitative information, de-convoluted FTIR spectrum of CS/HA-20 is provided in Figure 3.2(b), where the absorption peak at 1017 cm^{-1} indicates the presence of hydroxyapatite. The characteristic IR absorption bands, their assignments and corresponding peak positions for chitosan, hydroxyapatite, β -tricalcium phosphate and calcium pyrophosphate are reported in Table 3.1. XRD profiles of CS, HA and their nanocomposites are reproduced in Figure 3.2(c). HA peaks and their (h, k, l) indices are shown with black arrow heads. Similarly, XRD peaks for CS and intercalated CS layers are shown with red and pink arrows respectively. Broad XRD peak of CS at 2θ value of 22.8° is present in all composites along with the HA peaks.

Table 3.1. Characteristic IR bands positions for chitosan, hydroxyapatite, β -tricalcium phosphate and calcium pyrophosphate [199, 200]

Sample	Assignments	Wavenumber (cm^{-1})
Chitosan	$\nu(\text{-C=O})$ stretching	1718
	$\delta(\text{-NH})$ bending	1565
	$\delta(\text{-CH})$ bending	1344, 1376
	$\nu(\text{C-O-C})$ stretching	1152
	$\nu(\text{-C-O})$ stretching	1060, 1030
Hydroxyapatite	$\delta(\text{O-P-O})$ bending	558, 599
	$\nu_s(\text{P-O})$ symmetrical stretching	961
	$\nu_{as}(\text{P-O})$ asymmetrical stretching	1029, 1089
β -tricalcium phosphate	PO_4^{3-} stretching	940, 1188
Calcium pyrophosphate	$\text{P}_2\text{O}_7^{4-}$ stretching	725, 1213

A new XRD peak at around 16° observed mainly in CS/HA-10 and CS/HA-20 is attributed to the intercalated layers of CS. The intercalated CS peak, which is due to

penetration of HA nanoparticles between CS layers is clearly observable in SEM images of nanocomposites containing 10% and 20% by weight of HA nanoparticles.

3.3.2.2 Cross-sectional analysis

Cross-sectional SEM images of CS/HA nanocomposite films are provided in Figure 3.3(a, b, c, d). As can be seen here, SEM images of CS and CS/HA-5 films show the presence of fairly smooth surfaces. On the other hand, CS/HA nanocomposites containing 10 and 20% by weight HA clearly show the presence of a layered morphology, which is schematically depicted in Figure 3.3(h).

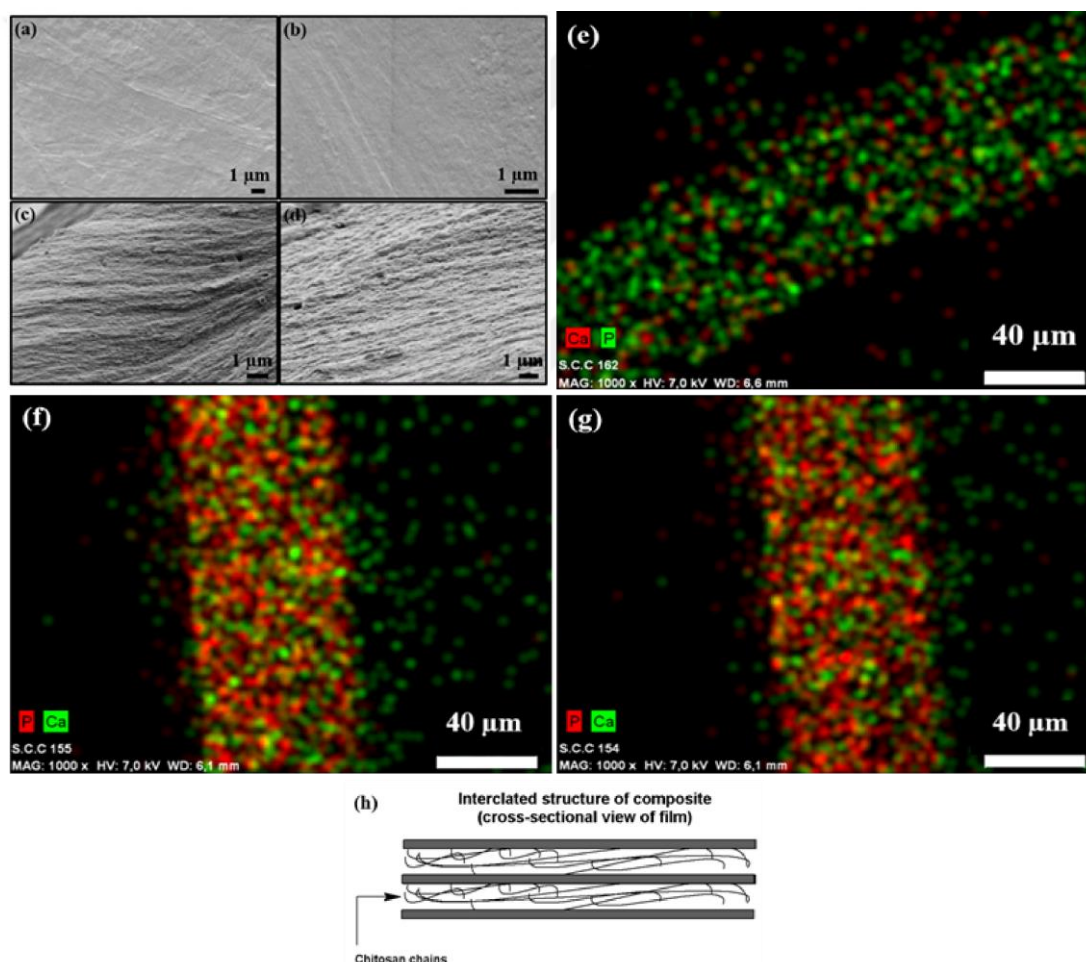


Figure 3.3. Cross-sectional SEM images of (a) CS/HA-0, (b) CS/HA-5, (c) CS/HA-10, (d) CS/HA-20; EDX mapping for Calcium and Phosphorous of (e) CS/HA-5, (f) CS/HA-10, (g) CS/HA-20 and (h) schematic description of the presence of a layered morphology in CS/HA nanocomposites

As will be discussed later on, presence of a layered structure is also demonstrated by the thermal decomposition studies of the nanocomposites, where upon complete degradation of CS matrix, layered porous HA structures were observed. Cross-sections of each composite was also analyzed through EDX mapping analysis. Characteristics X-rays generated from “Calcium” and “Phosphorus” were mapped and maps are presented in Figure 3.3(e-g). This EDX mapping strongly support our hypothesis that hydroxyapatite nanoparticles are uniformly distributed in throughout the films.

3.3.2.3 Surface analysis

Surface analysis of CS/HA composite films were performed by SEM and AFM. Prior to SEM analysis, samples were sputter coated with 10 nm gold layer. As shown in Figure 3.4(a), surface of CS film is fairly smooth. Presence of disc shaped HA nanoparticles on the surface of CS/HA nanocomposites, embedded into the CS matrix, can clearly be seen in the SEM images provided in Figure 3.4(b, c, d).

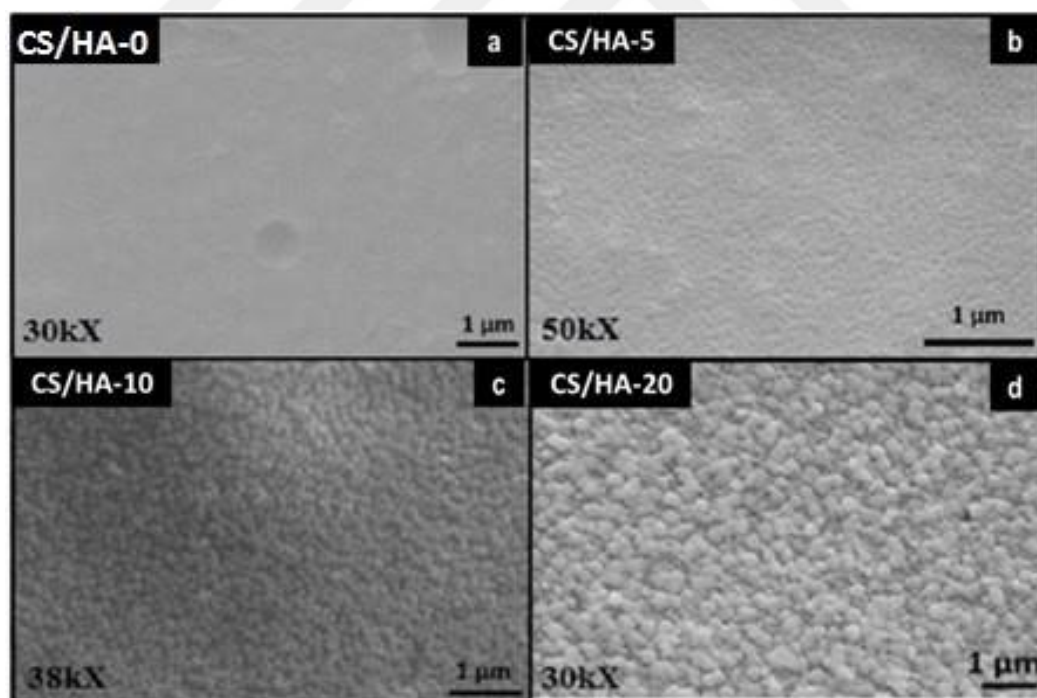


Figure 3.4. SEM surface images of (a) CS, (b) CS/HA-5, (c) CS/HA-10 and (d) CS/HA-20 nanocomposites

Nanoparticles present on composite surfaces exhibit very uniform disc like morphology and their sizes were analyzed through statistical image analysis by using ImageJ[®] tool. 25 randomly selected particles on the surface of each film were analyzed and sizes were found to be 44 ± 7 , 101 ± 16 and 229 ± 33 nm ($n=25$) for 5, 10 and 20% by weight HA content respectively. These dispersed HA particles are comparable in shape and size to the natural bone apatite particles [201]. In order to better understand the nature of the CS/HA nanocomposite surfaces, AFM analyses were also performed. $5\times5\text{ }\mu\text{m}^2$ AFM phase and height images for CS and CS/HA nanocomposite films are provided in Figure 3.5. As expected, AFM results are quite similar to SEM results.

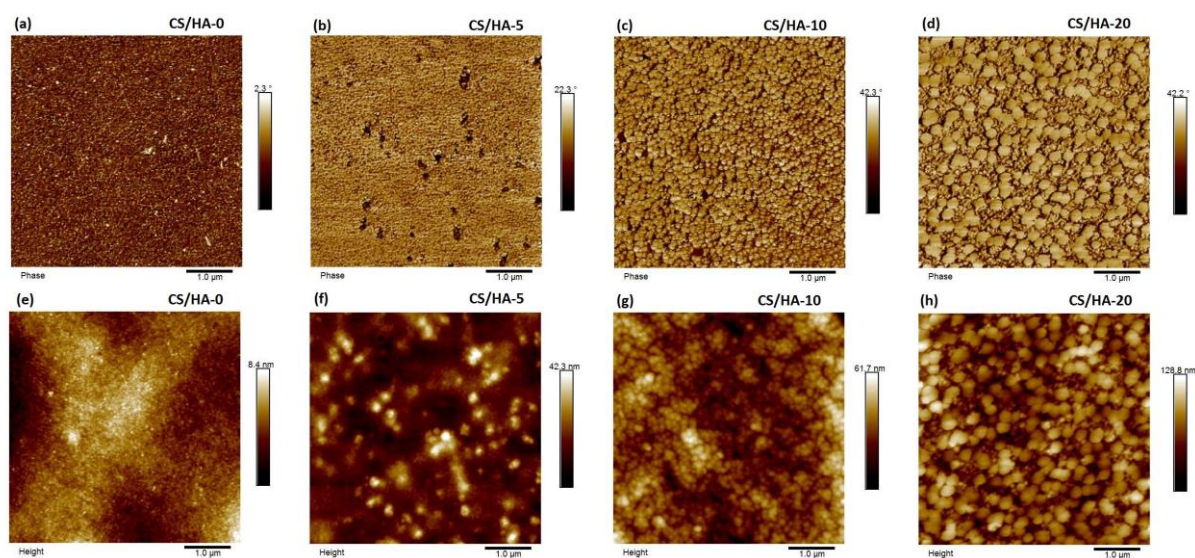


Figure 3.5. $5\times5\text{ }\mu\text{m}^2$ AFM phase and height images of CS and CS/HA nanocomposite films

Particle size distribution throughout the films at various concentrations is quite uniform as seen by the phase and height images of neat chitosan and CS/HA nanocomposites. It is observed that as the hydroxyapatite content of the nanocomposites increased, phase angle also increased, indicating increase in the surface hardness due to the presence of hydroxyapatite particles as shown in Figure 3.5. Phase angle in Figure 3.5(a-d) varies from pristine chitosan (2.3°) to composite having 20% HA nanoparticles (42.2°). Similarly height also varies from 8.4 nm (smooth film in case of chitosan only) to 128.8 nm (rough surface due to inorganic hydroxyapatite particles) as presented in Figure 3.5(e-h).

3.3.2.4 Thermal Degradation Studies

Thermal degradation behaviors of chitosan, hydroxyapatite and their nanocomposites were studied by TGA. All samples were pre-dried at 100 °C for 1 h before analysis. Samples were degraded under dry air flow with a rate of 60 mL/min and a heating rate of 10 °C/min up to 1000 °C. Thermal degradation curves obtained are reproduced in Figure 3.6(a). As expected, hydroxyapatite does not show any degradation up to 1000 °C. Thermal degradation of neat CS and CS/HA nanocomposites display composition dependent profiles.

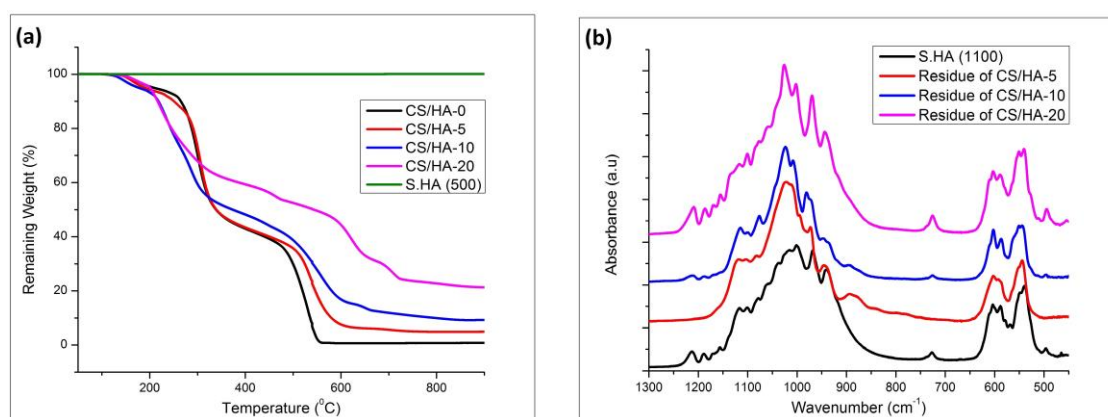


Figure 3.6. (a) TGA curves for HA, chitosan and CS/HA nanocomposites; (b) ATR-FTIR spectra for HA-1100 and TGA residues of CS/HA nanocomposites

All CS/HA nanocomposites start thermal degradation around 200 °C. It is interesting to note that CS/HA nanocomposites with higher HA loadings display a faster degradation rate up to about 300 °C. This may be due to better heat transfer to the CS matrix by the inorganic HA particles present on the surface, leading to faster degradation of the organic CS. After 300 °C, the trend changes and nanocomposites with higher HA content show slower degradation of the CS matrix. Complete degradation of CS matrix is achieved at 550 °C for neat CS, whereas for CS/HA-5, CS/HA-10 and CS/HA-20 complete degradation of the CS matrix is achieved at about 600, 700 and 750 °C, respectively. The residue amounts from TGA obtained for CS, CS/HA-5, CS/HA-10, CS/HA-20 and HA is 0.8, 4.9, 9.3 and 21.3 and 100% respectively, which consists of hydroxyapatite, calcium pyrophosphate and tricalcium phosphate. Amount of residue for each nanocomposite is

very close to that of original HA incorporated, clearly showing complete degradation of the CS matrix.

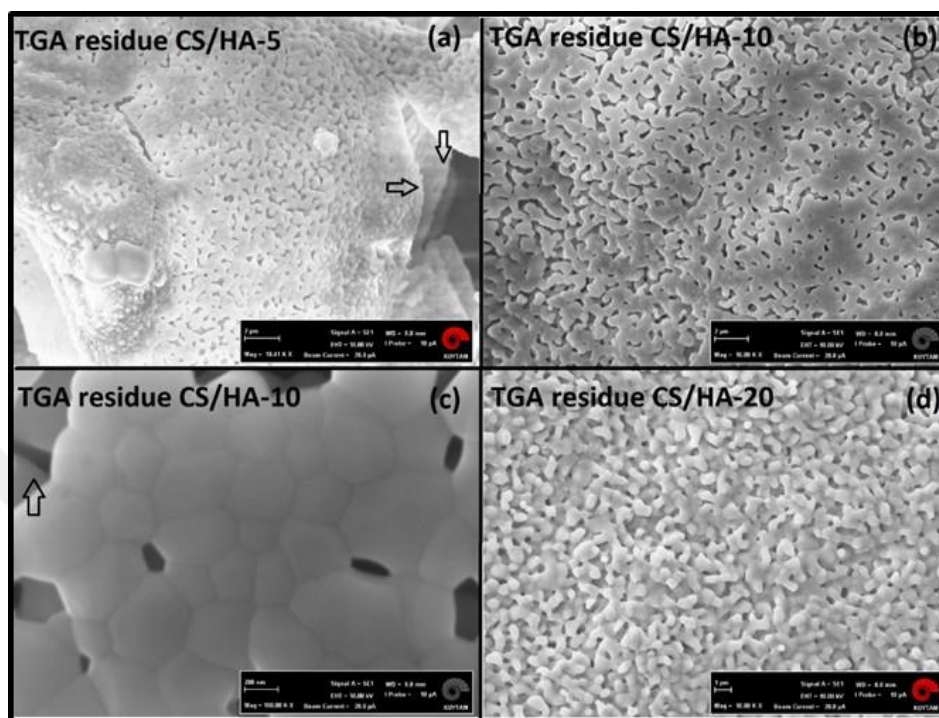


Figure 3.7. SEM analysis of residues obtained after thermal degradation of composites

We believe one of the most interesting observations resulted from the TGA study is the chemical and morphological investigation of the residues by ATR-FTIR, SEM and EDX. Figure 3.7 provides the SEM images of porous HA structures obtained after thermal degradation of CS/HA nanocomposites. As shown by arrows in the SEM images in Figure 3.7(a), for CS/HA-5 and Figure 3.7(c) for CS/HA-10, formation of layered porous 3D HA structures are clearly visible. Distribution of the pores with average sizes between 100 nm and about 1 μm are fairly uniform throughout these structures. As can be seen in Figure 3.7(c), where the SEM image of the residue obtained from CS/HA-10 is provided, some of the HA nanoparticles are fused together to form larger particles, which are a few hundred nm in size, with well-defined boundary layers. As clearly demonstrated by FTIR spectroscopy in Figure 3.6(b), these porous materials display identical spectra as that of HA-1000. Another important observation from these spectra is that in residues of CS/HA-10, CS/HA-20 and HA-1100, some phases have been converted to calcium pyrophosphate

as can be identified through their respective bands at 725 and 1213 cm^{-1} but no such bands were observed in case of porous films obtained from CS/HA-5.

3.3.2.5 Stress-strain analysis

Tensile properties of CS and CS/HA composites were determined by stress-strain analysis. Load versus strain behavior for all films was recorded and presented in Figure 3.8.

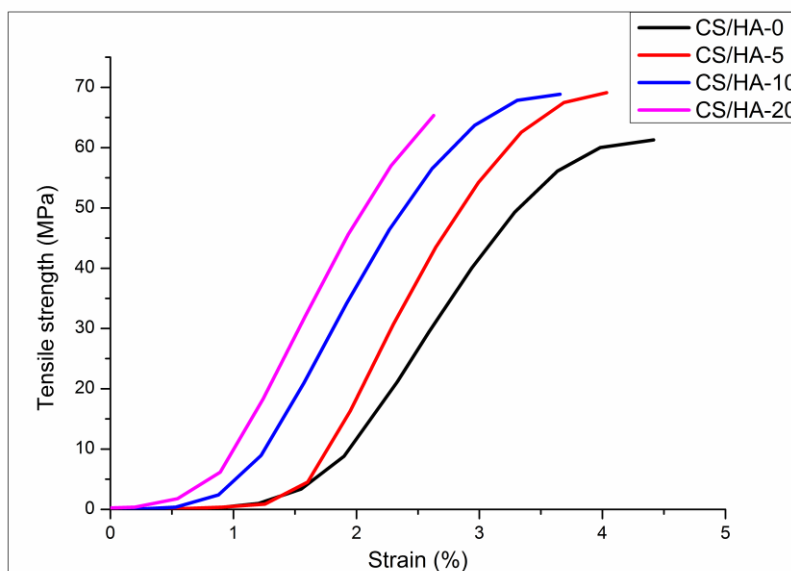


Figure 3.8. Stress-strain analysis of CS and CS/HA nanocomposites

Tensile strength was significantly increased from 61 MPa (pristine chitosan) to maximum of 69 MPa after incorporation of hydroxyapatite nanoparticles in composite films. There was no significant difference observed in the tensile strengths of composite films. Young's modulus increased from 3.5 GPa for CS to 4.4 GPa for CS/HA-20. On the other hand, elongation at break gradually decreased from 4.4% (chitosan) to 2.6% (CS/HA-20) as expected.

3.4 Conclusions

In this study, we synthesized hydroxyapatite nanoparticles through sol-gel process at low temperatures and at high yields. HA nanoparticles were used in the preparation of Chitosan/HA nanocomposites by solution casting. We introduced a novel solvent, pure

formic acid which yielded very stable dispersions of hydroxyapatite particles in CS solutions, for the preparation of CS/HA nanocomposites, containing 5, 10 and 20% by weight HA. SEM analysis on CS/HA film surface showed the presence of disc shaped HA nanoparticles with average particle sizes of 44 ± 7 , 101 ± 16 and 229 ± 33 nm for 5, 10 and 20% by weight HA content of the formic acid dispersions respectively. Formic acid produced stable dispersions of HA particles in CS solutions resulting in self-intercalated composite films as demonstrated by XRD, SEM and TGA studies. Chitosan chains were found to be in a layered structure in 10% and 20% HA containing films. Presence of nano-sized islands of HA on the surface of composite films along with embedded HA particles into the films was confirmed by different characterization techniques. After thermal degradation of these composites, layered porous structures consisting of hydroxyapatite, tricalcium phosphate and calcium pyrophosphate phases were obtained. These structures are quite uniform and well organized confirming our hypothesis of uniform and stable dispersion of nanoscale inorganic particles. CS/HA nanocomposites and microporous layered HA structures obtained can be considered as promising materials for bone tissue engineering applications since HA is known to promote adhesion, proliferation, and osteogenic differentiation of osteoblast like cells.

CHAPTER 4

PLA/CS/HA 3D COMPOSITE SCAFFOLDS DEVELOPMENT

4.1 Summary

Development of PLA scaffolds through fused deposition modeling and incorporation of chitosan (CS) and hydroxyapatite (HA) into these scaffolds to produce novel composite scaffolds is reported. Good mechanical properties of PLA, bioactivity of CS and osteogenic characteristics of HA are combined together to fabricate 3D scaffolds. PLA scaffolds are fabricated through low cost 3D printer. Scaffolds before and after incorporation of CS and HA were characterized through ATR-FTIR, SEM, EDX, water contact angle and XRD. Formic acid was used as the solvent to prepare CS/HA dispersions and found to be a good solvent for developing PLA/CS/HA composites. Strong interactions were observed between PLA, CS and HA. Surface properties of developed scaffolds were enhanced in terms of hydrophilicity, which resulted in significant improvement of U2-OS human osteosarcoma cells to attach and proliferate when compared to unmodified PLA scaffolds. 3D printed macro-porous PLA scaffolds were prepared according to pre-defined geometry. Due to high cost associated with high resolution printers, it is assumed that macro-porous scaffolds can be fabricated through ordinary printers and pores can be partially filled with bioactive materials that may also serve for improved cell attachment and their growth along with infiltration of by-products. PLA surface was immobilized through thin layer of chitosan and hydroxyapatite and macro-pores were partially filled with additives by utilizing proper solvent system. Formic acid was used as a solvent which dissolves CS and disperses HA particles well, producing stable dispersions of CS/HA. It also causes PLA chains to swell only, enabling CS chains to diffuse into PLA matrix, thus resulting in well prepared PLA/CS/HA composites. The interaction of CS, HA and PLA is evaluated through different characterization techniques. Schematics representation of process is shown in Figure 4.1.

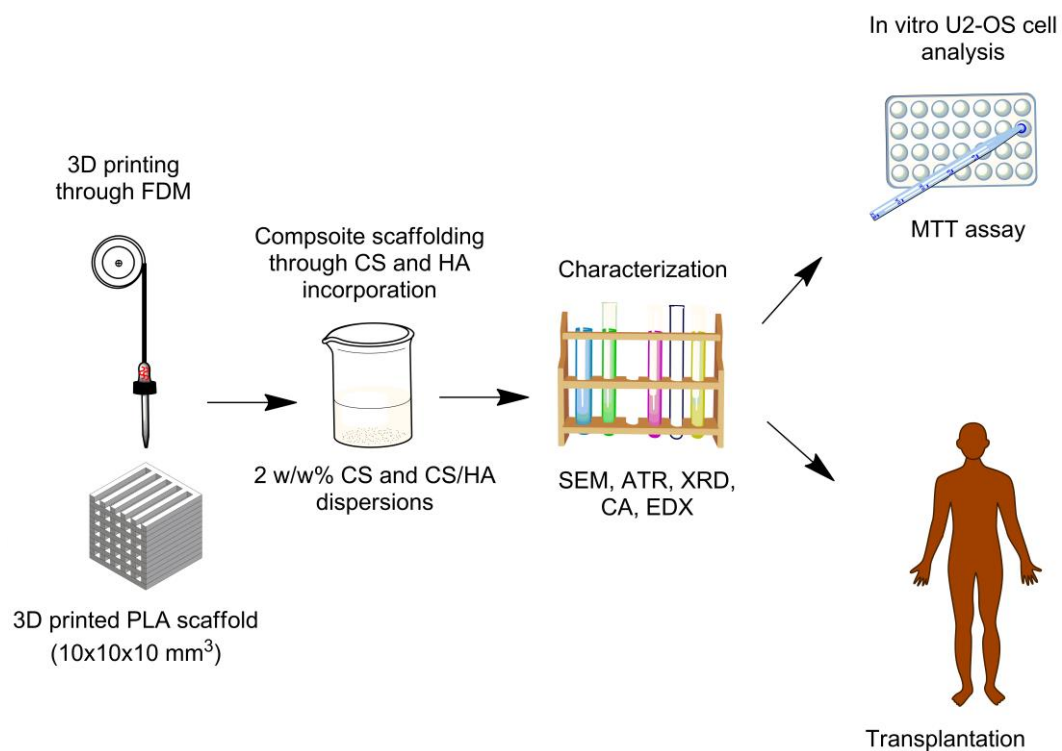


Figure 4.1. Schematic representation of PLA/CS/HA composite scaffolds development process

4.2 Materials and methods

4.2.1 Materials

PLA ($M_n=109,000$ g/mol, PDI=1.76 and 8% D content) was obtained from Natureworks (PLA polymer 4042D). Molecular weight was determined by GPC measurements using polystyrene standards. Low molecular weight chitosan having 75-85% degree of deacetylation was received from Aldrich. Formic acid (98%), diammonium hydrogen phosphate ($(\text{NH}_4)_2\text{HPO}_4$), calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$), ammonia solution (NH_4OH , 28-30%) and ethanol were used as received from Merck. Triple distilled water was used in all experiments. Nano-hydroxyapatite was synthesized in our laboratories. U-2 OS – Human osteosarcoma cell line (ATCC: HTB-96TM), Dulbecco's modified Eagle medium (DMEM-High Glucose) having 4.5 g/liter Glucose, fetal bovine serum, Penicillin/Streptomycin Solution, L -glutamine (Gibco, Grand Island, NY), 0.5% Trypsin-EDTA solution (Sigma-Aldrich, St. Louis, MO), Dulbecco's phosphate buffered saline (DPBS) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reagent (PanReac AppliChem) were used in all cell related experiments.

4.2.2 Methods

4.2.2.1 Hydroxyapatite synthesis

THF based synthesis described in Chapter 2, section 2.2.2.1 was used for developing composite scaffolds.

4.2.2.2 3D printed PLA and PLA/CS/HA composite scaffolds development

3D patterns were made in Delcam Powershape[®] and were extruded as stl. file. This model was processed in Simplify3D to generate g-code file. This g-code file was directly fed to Uzaras3D Dream Maker printer, a cheap alternative for making 3D scaffolds through fused deposition modeling equipped with heated bed. PLA filament was directly fed to the printer and scaffolds according to the required geometry were developed.

Low molecular weight chitosan (CS) was dissolved in formic acid as 2% w/w solution first, and followed by HA dispersion in CS solution as 10% and 20% on the weight of polymer. Formic acid was used during the preparation of these dispersions because it was found to be a good solvent for making stable dispersions of HA [185]. 3D PLA scaffolds produced were dipped in CS and CS/HA dispersions for five minutes, quenched in liq. N₂ and dried in vacuum oven at room temperature until constant weight. List of the samples prepared along-with their coding are provided in Table 4.1. These prepared samples were directly used for characterization and further analysis.

Table 4.1. Samples prepared, their coding and description

Number	Sample Code.	Description
1	PLA	3D printed scaffold
2	PLA+CS	3D printed scaffold dipped in CS solution
3	PLA+CS/HA-10	3D printed scaffold dipped in CS/HA (90-10) dispersion
4	PLA+CS/HA-20	3D printed scaffold dipped in CS/HA (80:20) dispersion

4.2.2.3 Characterization Techniques

Attenuated total Reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) analysis was performed on Thermo-scientific iS50 FTIR having ATR attachment. Absorption spectra were obtained by getting 64 scans at autogain with 2 cm⁻¹ resolutions. X-ray diffraction (XRD) analysis was performed on Bruker D8 Discovery X-ray diffraction system. X-ray (K α) generator composed of Cu, wavelength of 1.54184 Å at a voltage of 40 kV and 40 mA current was used for acquiring X-ray diffractograms. Morphological analysis of all specimens was performed on Zeiss ultra plus field emission scanning electron microscope (FESEM) having energy dispersive X-ray detector.

4.3 Results and Discussion

4.3.1 Characterization of PLA/CS/HA composites

4.3.1.1 ATR-FTIR analysis

ATR-FTIR spectra of all samples were acquired and are given in Figure 4.2. ATR-FTIR analysis of PLA, CS, HA and their composites. In case of PLA, -C=O band appears at 1746 cm^{-1} , after CS incorporation, this band shifted to 1716 cm^{-1} in all composites and also decreased in intensity due to chitosan present on the surface.

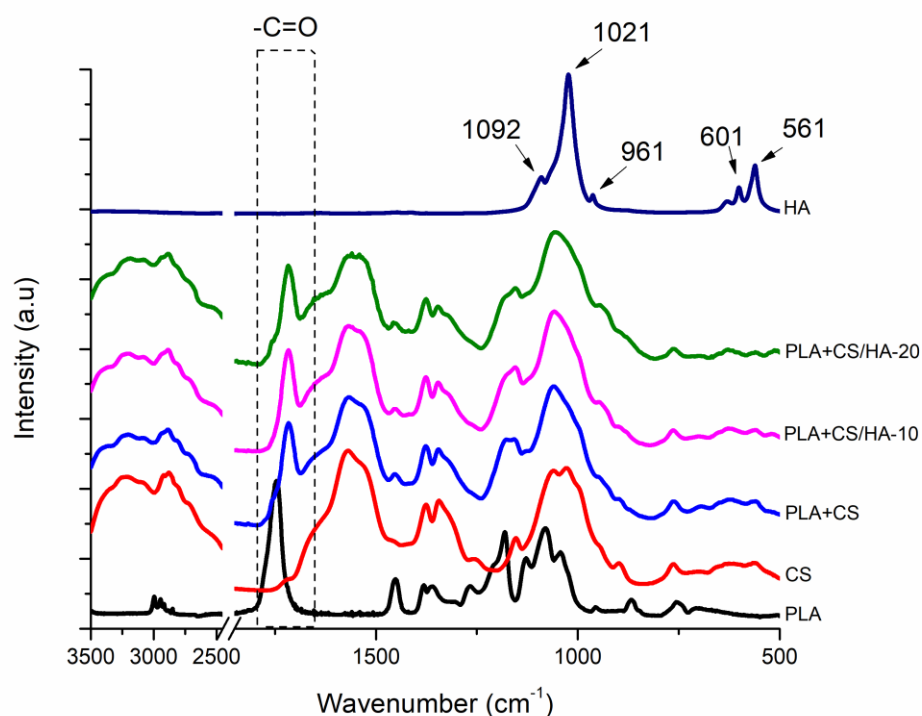


Figure 4.2. ATR-FTIR analysis of PLA, CS, HA and their composites

This shows that there is possible interaction existing between PLA and CS ($\text{C=O}\cdots\text{NH}$). N–H bending vibration band of chitosan appearing at 1568 cm^{-1} was also shifted to 1559 cm^{-1} in case of PLA+CS/HA-20. No shift is observed in PLA+CS and PLA+CS/HA-10. In case of PLA+CS, there was no HA and in PLA+CS/HA-10, little amount of HA was present. This implies that shifting in band of N–H is because of the interaction of HA particles with amino groups ($\text{PO}\cdots\text{NH}$). Corresponding bands of pure HA are shown in Figure 4.2. Bands at 561

and 601 cm^{-1} correspond to O–P–O bending while bands at 961, 1021 and 1092 cm^{-1} correspond to P–O symmetrical and asymmetrical stretching modes. Shift in HA bands is difficult to evaluate and quantify due to overlapping of chitosan bands in this region [185]. Overall, FTIR analysis concludes that there is successful incorporation of CS and HA in PLA scaffolds and strong interaction is present among CS, HA and PLA in composite scaffolds.

4.3.1.2 Surface analysis

Surface morphology of bare PLA and its composite scaffolds with CS and HA was investigated through SEM analysis and secondary electron images are shown in Figure 4.3. Figure 4.3(a, b, c) show the morphology of PLA scaffolds before CS and HA incorporation. Three different sides (canal like, plain surface and cubical structures) that were produced are shown. Figure 4.3(d, e, f) correspond to scaffolds after incorporation of CS, which clearly show the presence of chitosan on the surface. As can clearly be seen in Figure 4.3(f), pores were also partially filled with CS. Similarly, Figure 4.3(g, h, i) and Figure 4.3(j, k, l) represent scaffolds of PLA+CS/HA-10 and PLA+CS/HA-20 respectively. Further, strut-like morphology on plain side of samples in Figure 4.3(b) is not present anymore in Figure 4.3(e, h, k), which means that PLA surface was converted to smooth surface due to swelling in PLA chains and chitosan present on the surface of PLA. Samples having HA cannot be differentiated from samples having only CS. For its evaluation, EDX analysis was performed to verify the presence of HA particles in these composites. EDX analysis generated “Ca” and “P” X-rays and EDX mapping was performed. Results are given in Figure 4.4, where SE images, EDX mapping and EDX spectra showing signals for “Ca” and “P” are compared for PLA+CS/HA-10 and PLA+CS/HA-20 scaffolds. EDX spectra and mapping show clearly the X-ray signals coming from “Ca” and “P”. This certifies that HA particles are almost uniformly spread on the whole surface due to stable dispersion. As the surface hydrophilicity/hydrophobicity of scaffolds is also important for proper cell adhesion and proliferation, static water contact angle measurements were performed for all scaffolds produced. Samples were kept under vacuum prior to contact angle measurements.

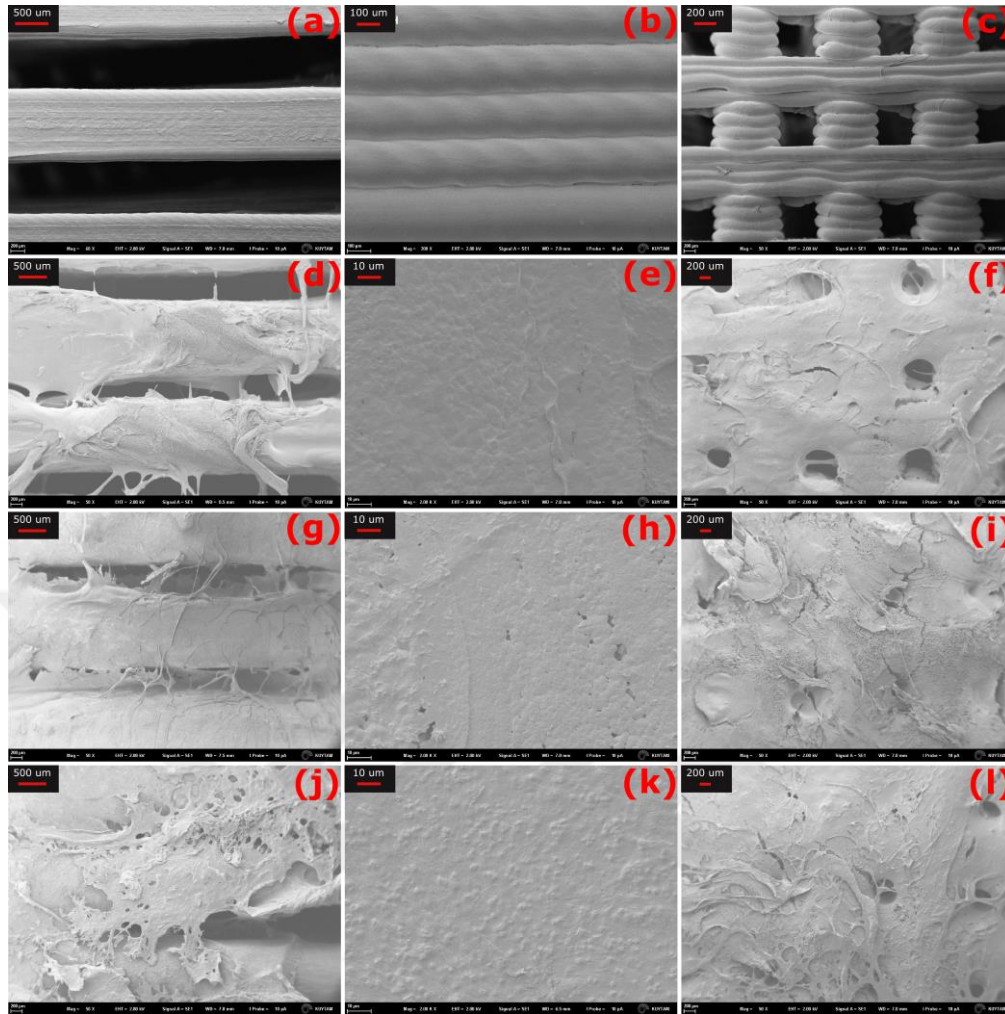


Figure 4.3. SEM analysis of PLA, PLA+CS, PLA+CS/HA-10 and PLA+CS/HA-20 scaffolds

10 μ L droplet of double distilled water was used for contact angle measurements. Contact angles for each sample along with images are shown in Figure 4.5. As, chitosan is hydrophilic, after incorporation of CS and HA the water contact angle considerably dropped from $80.2 \pm 3.4^\circ$ (PLA) to $38.1 \pm 2.9^\circ$ (PLA+CS/HA-20).

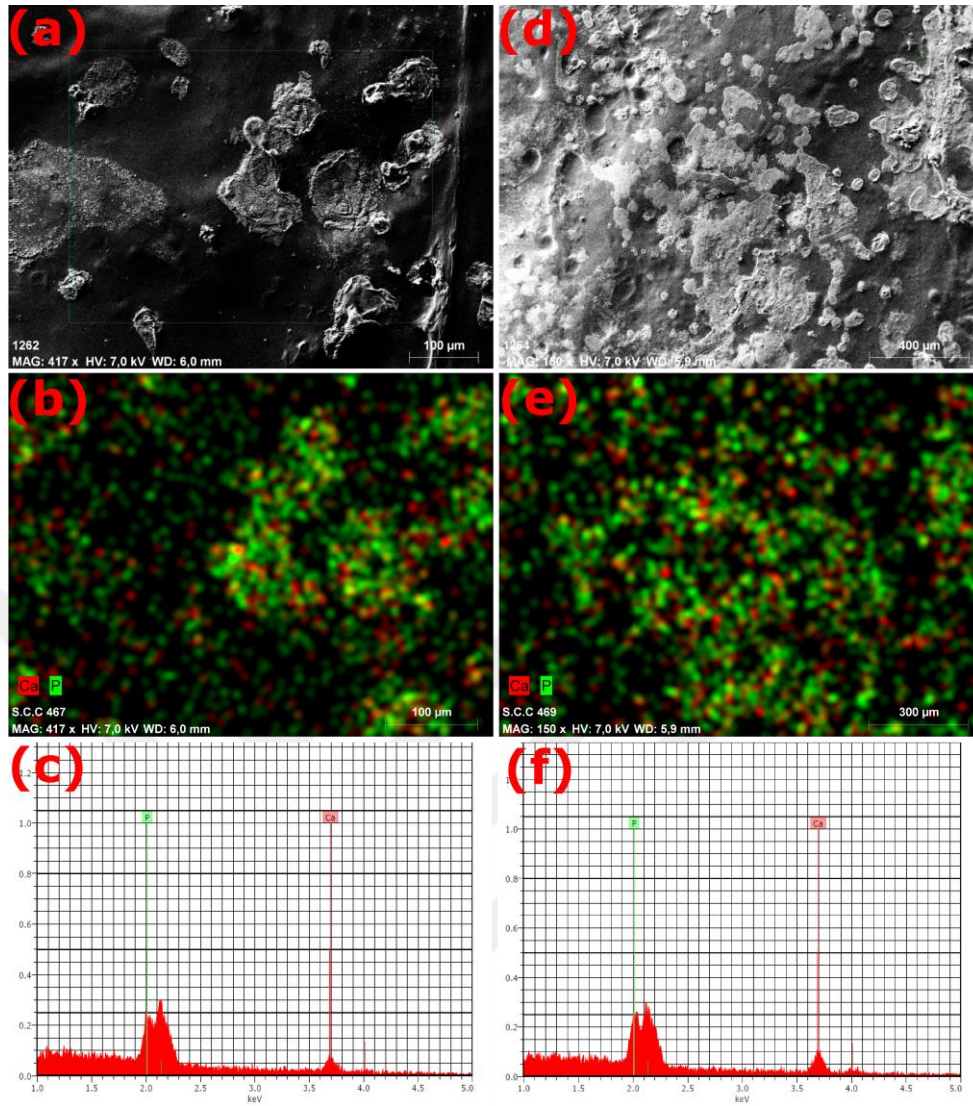


Figure 4.4. SE images, EDX mapping and spectra for: PLA+CS/HA-10 (a, b, c) and PLA+CS/HA-20 (d, e, f)

No significant difference was observed among PLA+CS ($43.2 \pm 0.8^\circ$), PLA+CS/HA-10 ($40.2 \pm 1^\circ$) and PLA+CS/HA-20 ($38.1 \pm 2.9^\circ$). We can say that, after incorporation of CS and HA in PLA scaffolds, surface became hydrophilic, which is preferable for cell attachment and growth [202].

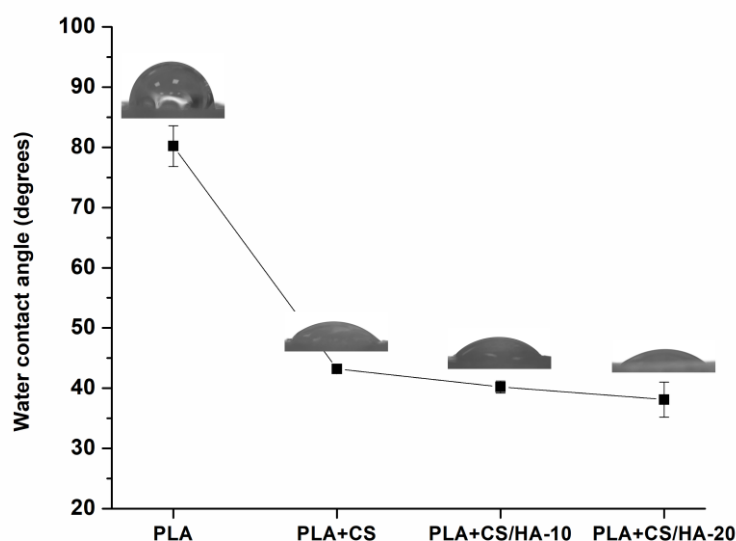


Figure 4.5. Static water contact angle measurement of PLA, PLA+CS, PLA+CS/HA-10 and PLA+CS/HA-20

4.3.1.3 XRD analysis

Crystallinity of the scaffolds produced by 3D printing were analyzed by XRD. Results are provided in Figure 4.6. XRD diffractograms of CS and HA are provided in inset figure. It is interesting to note that PLA scaffolds are amorphous, most probably due to fast cooling after printing, which does not provide long enough time for PLA chains to rearrange at room temperature to induce crystallinity. However, when CS and HA were incorporated from formic acid solutions, very interestingly PLA chains become crystalline, as can clearly be seen from two sharp peaks appearing between 2θ 15° and 20° in Figure 4.6. This observation also indicates that formic acid is a good enough solvent for PLA chains to swell and crystallize. Two major HA peaks in PLA+CS/HA-10 and PLA+CS/HA-20 composites are indicated in Figure 4.6. These peaks correspond to (0,0,2) and (2,1,1) planes of HA appearing at 26.3° and 31.3° respectively. These peaks in pure HA are present at 26° and 31.9° respectively. This very slight change can be due to interaction of HA particles with CS.

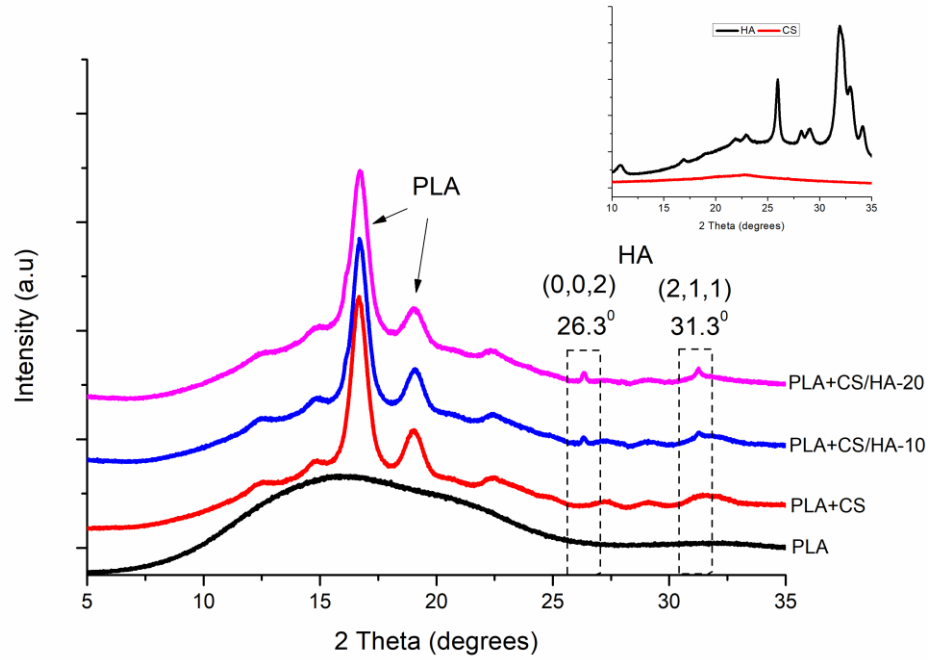


Figure 4.6. XRD diffractograms of PLA, CS, HA and their composites

4.3.1.4 Weight loss behavior of scaffolds in DMEM medium

To demonstrate the weight loss behavior of 3D scaffolds produced, they were subjected to a DMEM medium having 10% FBS. Before dipping, all scaffolds were dried under vacuum at room temperature for 24h. After weighing, scaffolds were completely immersed into the medium. The containers were then placed in an air oven at 37°C and samples were taken out after 1, 3, 7, 11 and 15 weeks. Samples taken out were rinsed with distilled water and then thoroughly washed with hot (50 °C) distilled water in order to remove any impurities like soluble products and salts from medium. Samples were weighed after drying in vacuum oven at 60 °C and percent weight loss was calculated by Equation 4.1.

$$\text{Weight loss (\%)} = \frac{W_t - W_i}{W_i} \times 100 \quad (\text{Equation 4.1})$$

Where “ W_i ” is the initial weight of scaffold before putting in medium and “ W_t ” is the weight of scaffold after taking out from medium at time “ t ”. W_t was recorded after washing and vacuum drying of samples completely. Medium was also changed at the same time interval of analysis for all samples. The remaining weight% results obtained are plotted in Figure 4.7.

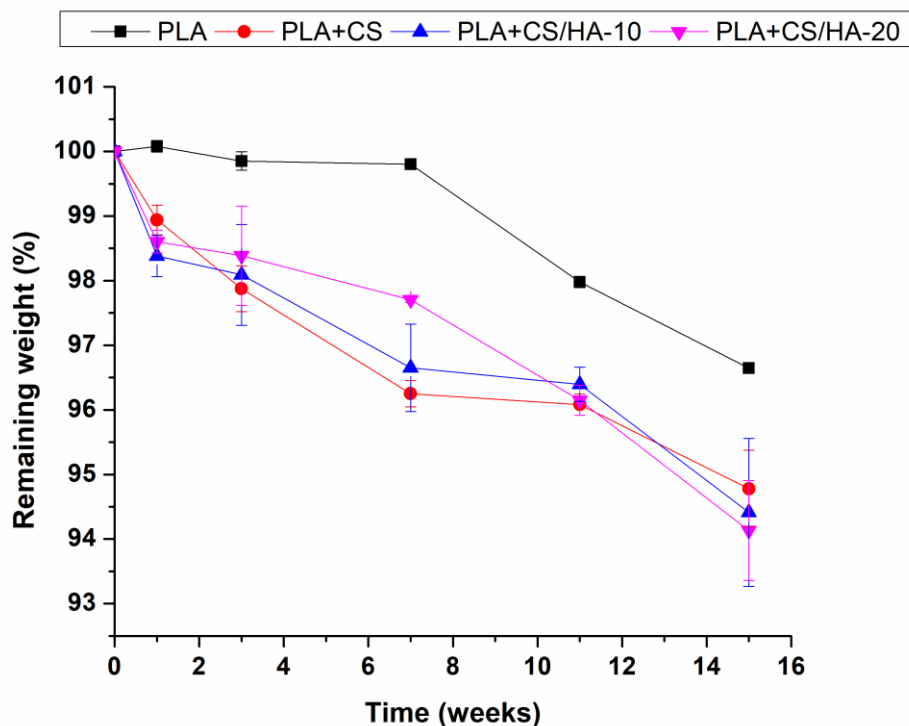


Figure 4.7. Weight loss behavior of PLA and PLA/CS and PLA/CS/HA scaffolds

PLA scaffolds exhibit a slow degradation rate in the medium used and displays about 3.4% weight loss after 15 weeks. This is most probably due to the hydrophobic nature of the PLA surface, which does not interact with the aqueous medium strongly. On the other hand, weight loss observed in scaffolds containing CS and HA is much faster and they lose more than 1% of their weight during the first week. This may most probably be due to the loss of CS and HA deposited on the surface, as well as inside pores. Composite scaffolds display ~5-6% weight loss at the end of 15th week, which is higher than PLA. This can be due to complete loss of CS and HA present on surfaces and partial degradation of PLA surface etched by formic acid. CS and HA present on surface can serve the purpose of initial attachment of cells and PLA can provide long-term mechanical support. Effect of CS and HA presence on cell attachment will be discussed in Figure 4.8. Overall results indicate that PLA/HA/CS composite scaffolds developed show fairly slow degradation that is desirable in bone tissue applications since bone takes much more time to regenerate so that this degradable matrix would replace with original bone ultimately. Cancellous bone takes 3-6 months while cortical bone takes 6-12 months for remodeling [203]. However, faster degradation was observed as a result of enzymatic degradation in simulated body fluid (SBF) resulting in 80% weight loss after 8 months [204].

4.3.1.5 *In vitro* cell viability

For cell viability analysis, U2-OS human osteosarcoma cell line was used. The cells were grown in 10% FBS, penicillin-streptomycin (5%), L-glutamine and DMEM media. Neutralized samples were sterilized from both sides under UV light for half an hour each and placed in a 24-well plate. In the meanwhile, medium in 100 mm plate was aspirated and the plate was washed with 5 mL of 1X PBS. After the cells were detached by using 1 mL of 0.5% Trypsin-EDTA solution, incubated for 2 minutes at 37 °C, 4 mL of DMEM low glucose (10% FBS, 1%P/S) was added into the plate and mixed with cells. Then 10 μ L sample of this mixture was pipetted out of the plate and poured in a hemocytometer. While the cells were counted under inverted microscope with 10X magnification, rest of the sample was taken to a falcon tube and centrifuged at 250g for 5 minutes. Supernatant in the falcon tube was aspirated and the medium amount required to re-suspend pellet was calculated to be 35,000 cells per well. The pellet was dissolved with calculated amount of DMEM high glucose (10%FBS, 1% P/S) and 500 μ L volume of re-suspended cells containing medium was seeded in triplicate into samples containing wells and control. Plates were incubated at 37 °C in a humidified 5% CO₂ atmosphere culture incubator.

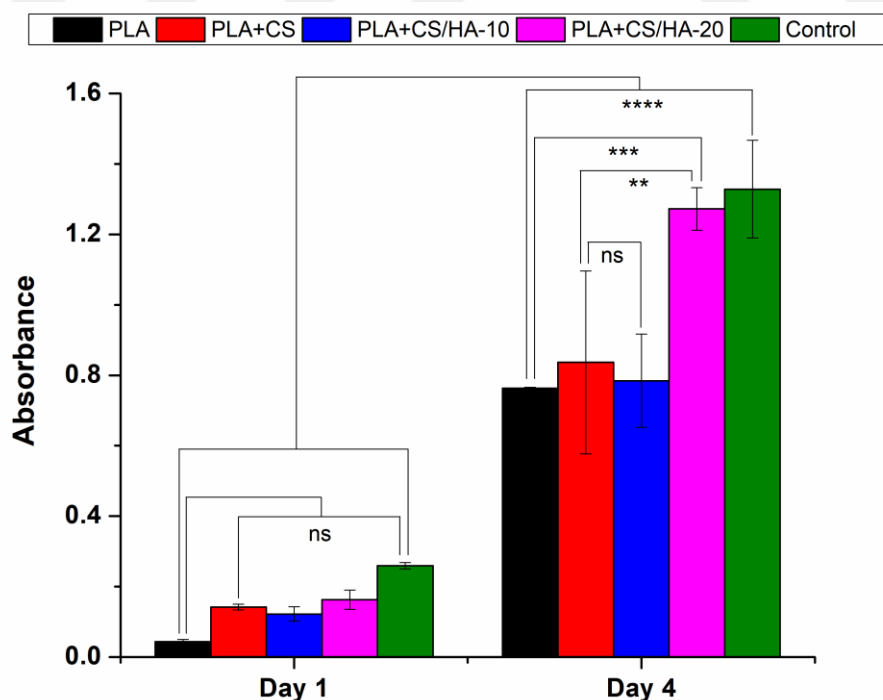


Figure 4.8. MTT analysis of scaffolds after 1st and 4th day of osteosarcoma cell seeding

The cytotoxicity was determined using an MTT assay at Day-1 and Day-4 (cell seeding on the materials are marked as Day-0). MTT stock solution was prepared as 1 mg/mL in complete medium. First, the medium in the plates was aspirated, then, 500 μ L of this solution including MTT (0.5 mg/mL) and DMEM at 1:4 ratios was added to each well. After this, cells were incubated in a humidified atmosphere of 5% CO₂ in air at 37 °C for 3.5 hrs. After the incubation period, the supernatant was removed, the dark blue formazan crystals were dissolved in 200 μ L mixture of ethanol and dimethyl sulfoxide (DMSO) (1:1 ratio). This solution was transferred into a 96-well plate, and the optical densities were read at 600 nm and 690 nm (the background absorbance) in Biotek Synergy plate reader. MTT analysis results are given in Figure 4.8. Two-way ANOVA analysis followed by Tukey's multiple comparison was performed. The results show that all the samples are non-toxic to cells due to proliferation and significant increase in number of cells from Day-1 to Day-4 (**** $p < 0.0001$). Day-1 analysis shows that after incorporation of CS and HA in scaffolds, the instant growth of cells was more than PLA scaffold. On Day-4, there was significant difference observed in composite scaffolds especially in case of PLA+CS/HA-20 than PLA (*** $p < 0.001$) and proliferation of cells is comparable to control. As a conclusion to MTT results, we can say that all the samples are non-toxic to cells and composite scaffolds offer better platform for cells to migrate and proliferate.

4.4 Conclusions

In this study, we developed PLA scaffolds through fused deposition modeling by using a simple desktop 3D printer. Due to the high cost associated with high resolution printers, we have demonstrated in this work that scaffolds possessing macro-porosity can be prepared by simple 3D printers. These pores can be filled by some bioactive materials like chitosan and hydroxyapatite. These scaffolds can be tailored according to the bone defect size. Scaffolds before and after CS and HA incorporation were characterized and evaluated. Solvent chosen (formic acid) was found to be suitable for incorporating CS and HA in porous PLA scaffolds. Cells were seeded on these scaffolds and results show that bone cells are able to attach and proliferate at higher rate on composite scaffolds than neat PLA scaffolds. So conclusively, these composite scaffolds can be promising materials for bone defects repair in tissue engineering applications.

CHAPTER 5

SILK COCOONS DEGUMMING AND SILK FIBROIN PROCESSING

5.1 Summary

In this part of my thesis, I investigated the degumming of silk (*Bombyx mori*) cocoons by using sodium carbonate (Na_2CO_3), sodium bicarbonate (NaHCO_3) and aqueous ammonia or ammonium hydroxide (NH_4OH) solutions. Degumming by carbonate and bicarbonate solutions are well established processes so they were used as controls, where cocoons were treated for 45 minutes in boiling aqueous solutions. In order to investigate the effect of degumming using ammonia solution, which is a novel and milder process, cocoons were subjected to treatment for 45, 60 and 90 minutes in boiling aqueous ammonia solutions. After washing and drying, degummed fibers were dissolved in phosphoric acid/formic acid (7/3) (v/v) mixture and coagulated in methanol. Pure silk fibroin was produced after washing and drying, without the need for dialysis. Coagulated and dried silk fibroin powder was re-dissolved in formic acid and films were cast. Degummed fibers and silk fibroin films were characterized by various techniques, which included scanning electron microscopy, (SEM), infrared spectroscopy (ATR-FTIR), x-ray diffraction (XRD) and thermogravimetric analysis (TGA). The complete process involving novel degumming through ammonia solution and comparison with other procedures and dialysis free silk fibroin film fabrication is illustrated in

.

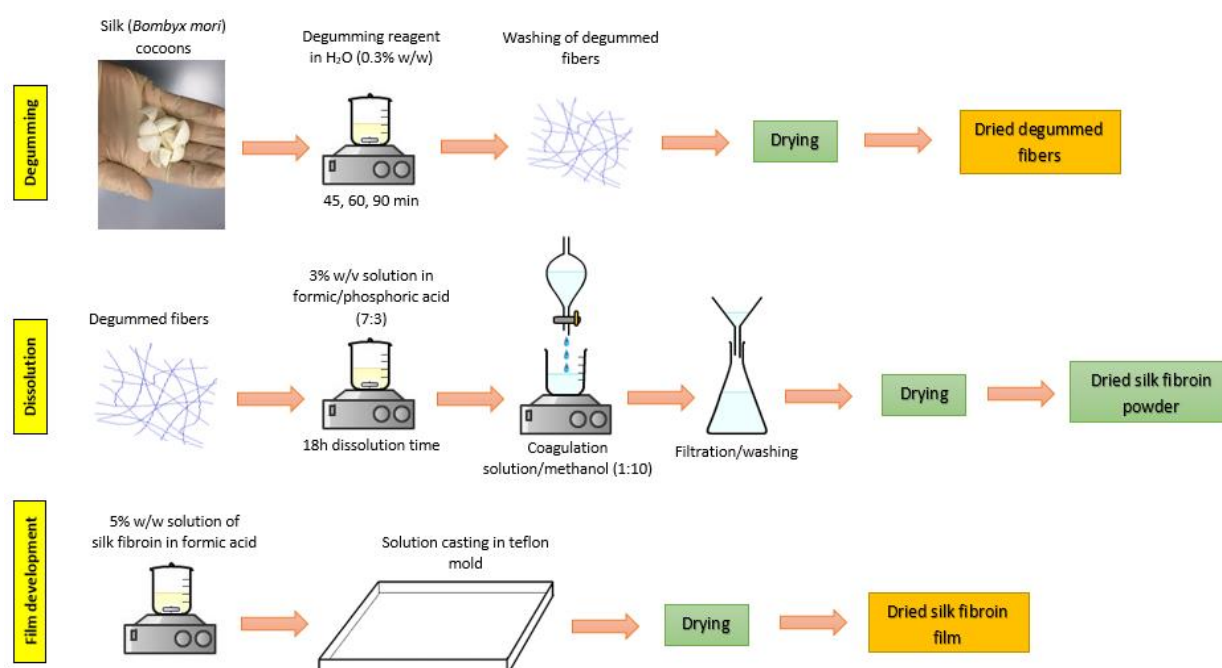


Figure 5.1. Schematic representation of the process steps used for degumming and recovery of silk fibroins and preparation of film from silk fibroin

5.2 Materials and Methods

5.2.1 Materials

Silk (*Bombyx mori*) cocoons were kindly supplied by Idealab, Koc University, Istanbul. Reagent grade sodium carbonate, sodium bicarbonate, ammonia solution (28-30%), methanol, formic acid (98%) and phosphoric acid were received from Merck and were used as received. Deionized and double distilled water was used in all solutions.

5.2.2 Methods

5.2.2.1 Silk cocoons degumming

Silk cocoons were degummed with minor modifications to the procedure described in the literature [205]. Briefly, 6 grams of the reagent was dissolved in 2000 mL of double distilled water to make 0.3 w/w solutions of sodium carbonate, sodium bicarbonate and ammonia. For comparison purposes, the concentration of ammonia solution was also kept the same as the other solutions, only processing time was varied. The solutions were heated and stirred. When solution started boiling, 5 grams of cut and cleaned pieces of cocoons were added into the beaker. Cocoons were treated in boiling sodium carbonate (Carbonate-45) and sodium bicarbonate (Bicarbonate-45) solutions for 45 minutes in. In case of ammonia solutions, cocoons were treated for 45 (Ammonia-45), 60 (Ammonia-60) and 90 minutes (Ammonia-90). After treatment, degummed fibers were washed in freshly distilled water and rinsed until the water became neutral. The fibers were dried at room temperature overnight and then in a vacuum oven at 40 °C until constant weight.

5.2.2.2 Dissolution of degummed fibers

3% (w/v) solutions of degummed fibers in formic acid/phosphoric acid (7/3) (v/v) mixture were prepared by magnetically stirring for 18 h at room temperature. After complete dissolution, silk fibroin was coagulated in methanol under constant stirring and filtered using a filter paper (Filter-lab REF.1525). Methanol to silk fibroin solution ratio was 10:1.

Coagulated silk fibroin residues were repeatedly washed with distilled water until the filtrate became neutral. Silk fibroin was dried in a fume hood at room temperature overnight and then in a vacuum oven at 40 °C for 24 h.

5.2.2.3 *Preparation of silk fibroin films*

Coagulated and dried silk fibroin was dissolved in formic acid to prepare 5% (w/w) solutions. Solutions were cast in Teflon® molds and after evaporation of formic acid, silk fibroin films were obtained. The films were characterized by ATR-FTIR, XRD, XRD, AFM and water contact angle measurements.

5.2.3 *Characterization techniques*

ATR-FTIR spectra of silk cocoons, degummed fibers and silk fibroin films were obtained on a Thermo-scientific iS50 FTIR spectrometer with an ATR attachment. Absorption spectra were recorded at a resolution of 2 cm⁻¹ and 64 scans. X-Ray Diffraction (XRD) patterns of cocoons, fibers and films were acquired on a Bruker D2 X-Ray Diffractometer. Scanning was made for 2θ of 10-90° in a total of 3920 steps with 10 seconds per step. Cu X-rays generator with a wavelength of 1.54184Å at a voltage of 30kV and 10mA current was used. Surface images of all specimens were obtained on Zeiss EVO LS 15, Scanning Electron Microscope (SEM). Samples were coated with 10 nm of gold layer through sputtering before the measurements. Atomic force microscopic analysis was performed on a Bruker Dimension Icon AFM. Phase and amplitude error images were obtained through standard tapping mode by using Bruker MPP-11120-10 (RTESPA model) tip with a force constant of 40 N/m, resonant frequency of 300 kHz and back side coated with 50±10 nm aluminum. Thermogravimetric analysis (TGA) was carried out on a TA-50 WSI instrument. Samples were heated up to 800 °C with a heating rate of 10 °C/min under 20 mL/min air flow. Contact angle measurements were performed on dataphysics OCA 35 goniometer by using a 5 µL droplet of distilled water.

5.3 Results and discussions

5.3.1 Characterization of degummed silk fibers

5.3.1.1 SEM analysis

Surface SEM images of silk fibers obtained before and after degumming by using different reagents are reproduced in Figure 5.2. For comparison, Figure 5.2(a) shows SEM image of silk cocoon fibers. SEM images of degummed and sericin free silk fibroin fibers obtained by different processes are provided in Figure 5.2(b-f).

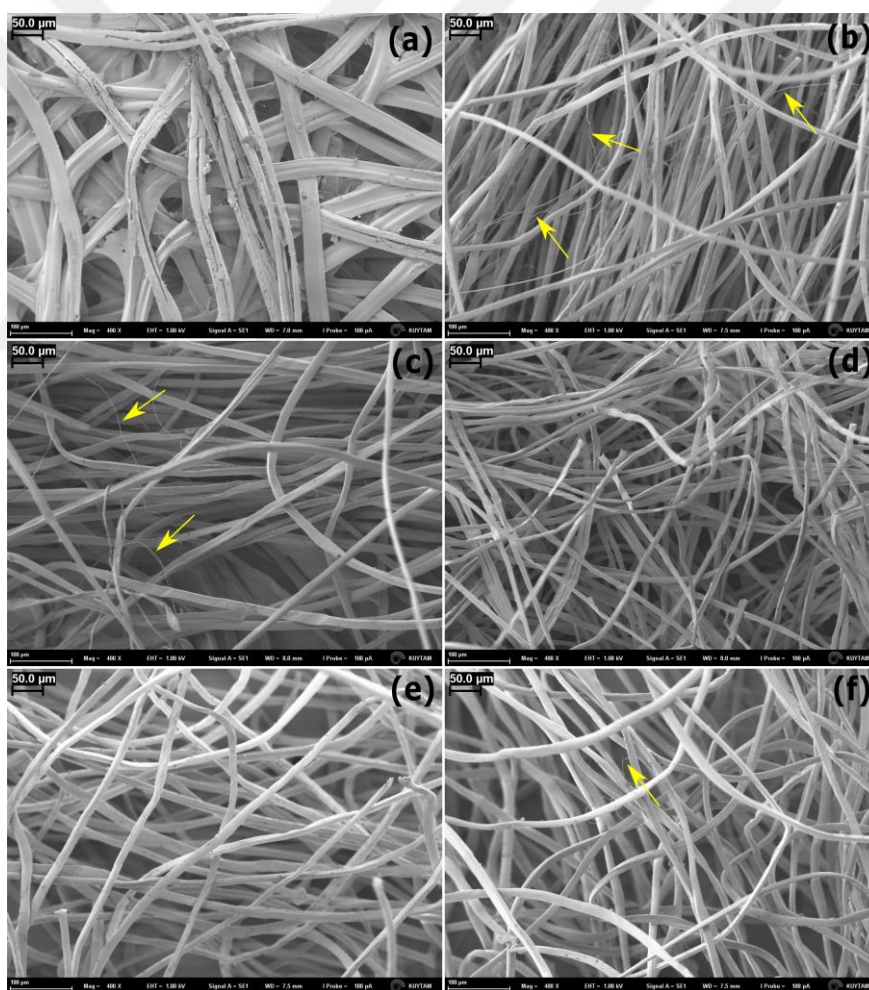


Figure 5.2. Surface SEM images of cocoons and degummed fibers: (a) cocoon fibers, (b) Carbonate-45, (c) Bicarbonate-45, (d) Ammonia-45, (e) Ammonia-60, and (f) Ammonia-

Reagent type and degumming time has significant effect on the surface morphology of silk fibroin fibers. Harsh chemicals and/or prolonged degumming time may cause serious damage to the fiber surfaces as can be seen in Figure 5.2(b, c, f). Sodium carbonate has already been proven to be fairly damaging to fiber's surface [206]. The fiber surfaces are prone to surface hydrolysis by the base solutions used, which ultimately causes fibrillation as indicated by the yellow arrows in Figure 5.2. There was no surface deterioration when the fibers were degummed through Ammonia-45 and Ammonia-60 processes. Less damage on the surface was observed for Ammonia-90 but more for Carbonate-45 and Bicarbonate-45. These results clearly demonstrate the superiority of ammonia degumming, developed in this study compared to conventional carbonate and bicarbonate processes.

5.3.1.2 ATR-FTIR analysis

3500-3200 cm^{-1} and 1800-1200 cm^{-1} regions of the FTIR spectra of cocoons and degummed fibers are provided in Figure 5.3. As expected, in all samples very sharp and symmetrical hydrogen bonded amide (N-H) absorption band is observed at 3280 cm^{-1} . Detailed structural information can be obtained from 1800-1200 cm^{-1} region, where absorption bands for Amide-I, Amide-II and Amide-III are observed. 1720-1580 cm^{-1} is the Amide-I region, where free and hydrogen bonded C=O stretching bands are observed. Amide-II region (1580-1480 cm^{-1}) comprise of C-H stretching and N-H bending vibrations, whereas the Amide-III region (1300-1200 cm^{-1}) provides in-phase combinations of C=O bending and N-H stretching vibrations [207]. In Amide-I region, absorption peaks for strongly hydrogen bonded and well-ordered amide groups ($\text{C=O}\cdots\text{H-N}$) that form the β -sheet structure appear at 1698 and 1617 cm^{-1} . Especially the strongly hydrogen bonded carbonyl peak centered at 1698 cm^{-1} is much sharper in the degummed fibers when compared with the untreated cocoons, due to sericin removal. In Amide-II region, peaks at 1505 and 1555 cm^{-1} correspond to β -sheet conformation and the peak at 1527 cm^{-1} is of the random coil, which overlaps with the strong (C-C) stretching peak at 1513 cm^{-1} [208]. In Amide-III region, peak for β -sheet and α -helical appear at 1260 and 1227 cm^{-1} respectively [209]. In case of cocoons, the weak shoulder at $\sim 1650 \text{ cm}^{-1}$ is most probably due to water soluble sericin, which is not present in degummed fibers. These

FTIR results clearly indicate that regardless of the procedure and the chemicals used, degummed silk fibroin fibers show very ordered and crystalline structures, which is also supported by the XRD results.

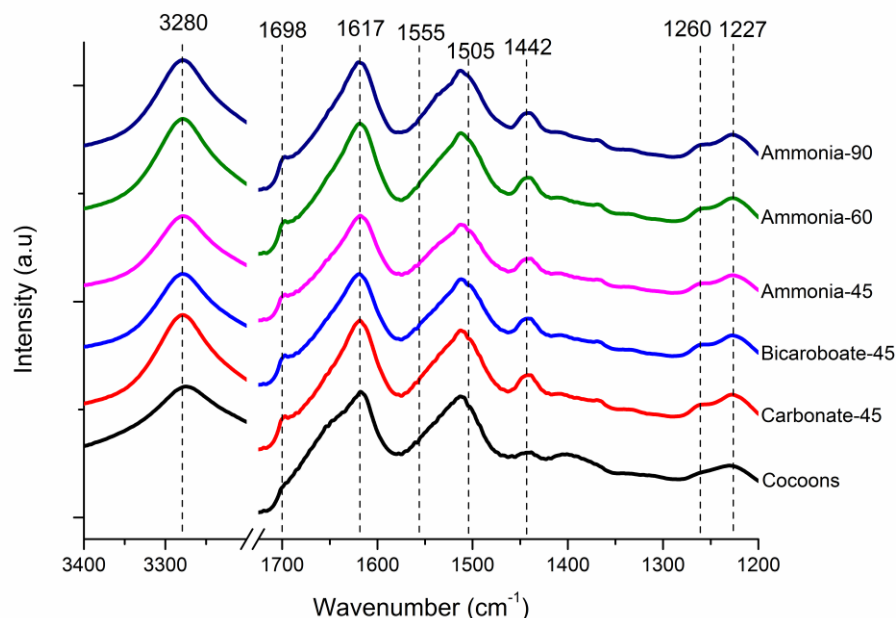


Figure 5.3. Comparative FTIR spectra of cocoons and degummed fibers obtained by using various processes

5.3.1.3 X-rays diffraction analysis

XRD characterization was employed to examine the crystallinity of the fibers before and after degumming process. Samples were analyzed for ~11 hours. X-ray diffractograms obtained are reproduced and compared in Figure 5.4. As the degradation becomes severe during degumming process, Silk-I and amorphous structure tends to dominate. In Figure 5.4., we can see silk-I (α -helical) and silk-II (β -sheet) structural peaks at $2\theta=29.2$ and 20.9° , respectively. In case of virgin cocoons, a well-defined and sharp silk-II XRD peak at $2\theta=20.9^\circ$ and a small shoulder-like, Silk-I peak at $2\theta=29.2^\circ$ are present. The sharp peak of Silk-I conformation tends to broaden as the cocoons are treated in basic media for sericin removal. Although this effect can be observed in all diffractograms of degummed fibers, the effect seems to be minimal for samples subjected to 45 min treatment in carbonate, bicarbonate and ammonia. For prolonged treatment (ammonia-60 and ammonia-90), much

broader peaks are observed, which are typically characteristic diffraction patterns for amorphous silk as indicated by circles in Figure 5.4. These results clearly demonstrate that treatment time has more prominent effect on silk fibroin conformation than the type of the weak base used. Thermogravimetric analyses of cocoons and degummed fibers were also performed and presented as Appendix III. No significant differences were observed on the thermal degradation behavior of cocoons or fibers degummed by using different reagents and conditions.

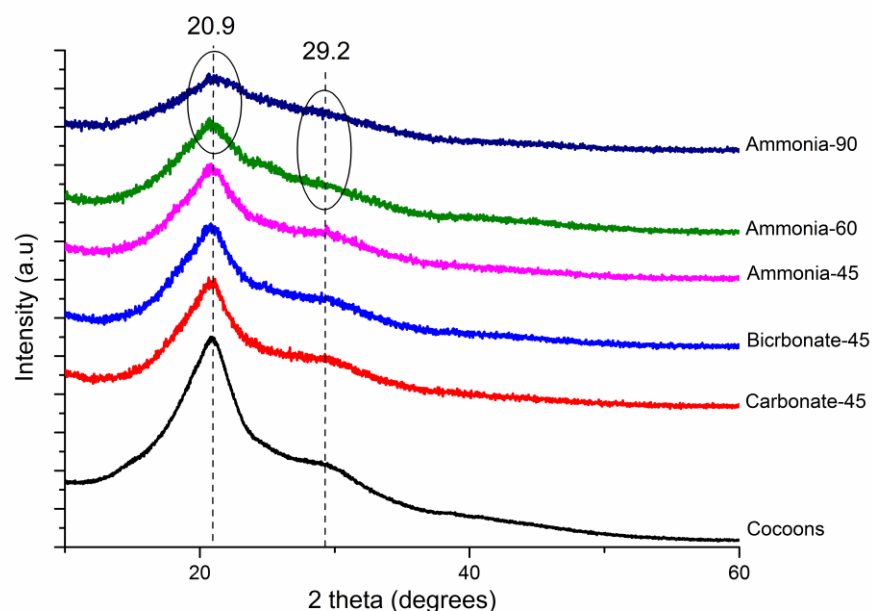


Figure 5.4. XRD analysis of cocoons and degummed fibers

5.3.2 Characterization of silk fibroin films

Silk fibroin films were obtained after dissolving the degummed fibers in phosphoric acid/formic acid (7/3) (v/v) mixture followed by coagulation in methanol, filtration/drying and re-dissolution of dried silk fibroin powder in formic acid as schematically explained in Figure 5.1. Films were then treated by soaking them in methanol/water (4/1) (v/v) solution for 1 h. Films were characterized before and after methanol treatment by ATR-FTIR, XRD, TGA, AFM and water contact angle measurements.

5.3.2.1 ATR-FTIR analysis

Crystallization of silk fibroin involves conformational transition and this transition can be easily induced through physical, chemical or mechanical treatment. Silk-I (α -form) can be converted to more stable silk-II (antiparallel β -sheet) by physical shear or exposure to organic solvents, such as methanol. Treatment with water favors silk-I, whereas exposure to methanol promotes silk-II formation [210]. Therefore, we treated our films with methanol/water mixture and checked out the influence of this treatment on films obtained through various processes. Silk fibroin films before and after methanol/water mixture treatment were characterized through ATR-FTIR as well. Amide-I, amide-II and amide-III regions were investigated and results are reproduced in Figure 5.5. FTIR absorption spectra of film obtained from carbonate-45 and ammonia-45 based treated degummed fibers. Spectra for other films like bicarbonate-45, ammonia-60 and ammonia-90 are given as Appendix IV.

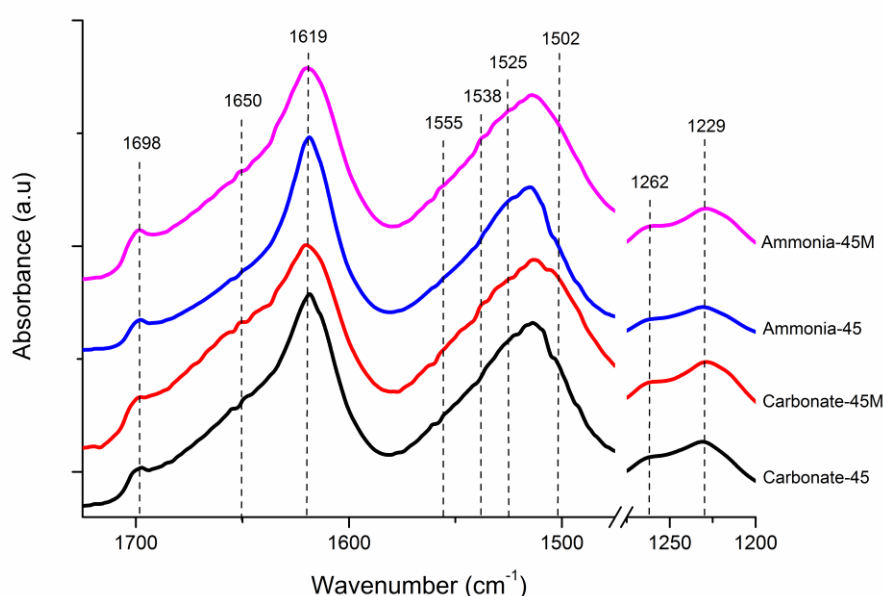


Figure 5.5. ATR-FTIR analysis of silk fibroin films before and after methanol treatment

According to the literature [211-213], IR absorption bands for Silk-I, Silk-II and amorphous structures in as cast silk fibroin films are not influenced by protein side chains

significantly so they appear at their corresponding wavenumbers as shown in Figure 5.5., although some of the absorption peaks are quite weak. Different functional groups responsible for Silk-I, Silk-II and amorphous configurations in Amide-I, II and III regions are already well explained in Figure 5.3. FTIR spectra of silk fibroin films can be used to quantify Silk-I and Silk-II content by the deconvolution of the amide peaks. For this purpose, Amide-I region ($1705\text{--}1595\text{ cm}^{-1}$) was processed in OMNIC[®] software. OMNIC peak resolve is based on Fletcher-Powell-McCormick algorithm and Gaussian line is used for solid samples [214]. So, peaks were found by using Gaussian line shapes through low sensitivity. Peaks were fitted by selecting constant baseline with a noise target of 10. These analyses provided very interesting results revealing the influence of degumming conditions on the conformational composition of silk fibroin films. Hu et al. used FTIR bands in Amide-I region at 1611 cm^{-1} for side chains and $1619, 1624, 1630, 1698\text{ cm}^{-1}$ for β -sheet. They assigned bands at $1640, 1650\text{ cm}^{-1}$ for random coils and $1666, 1680, 1691\text{ cm}^{-1}$ for β -turns. Band at 1659 cm^{-1} was assigned to α -helical [211]. Rajkhowa and co-workers [215] also used the same bands for deconvolution to determine the conformation in silk fibroin films. We also used these bands assigned for β -sheet, α -helical, β -turns, random coil and side chains. Resolved peak data for each sample is provided in Table 5.1, while the spectra after deconvolution are provided as Appendix-V.

Conventionally aqueous solutions of silk fibroin obtained after dialysis are cast to produce films. Such films display mostly amorphous structure together with some Silk-I structure. When films are treated with water, Silk-I predominates. While upon methanol treatment, Silk-II prevails. During water treatment, a skin-like thin film layer is formed at the air-water interface. This acts as a barrier resulting in fairly slow evaporation of water. Slow evaporation provides ample time to the silk fibroin chains to self-assemble, leading to significant increase in silk-I structure [210]. In our study, the films were cast from formic acid solutions, in as cast films, where such a skin film did not form. Consequently, films cast from formic acid solution possess more Silk-II structures due to the possible differences involved in drying kinetics. Silk-II content of 46.2%, 33.9% and 35.9% was observed in ammonia-45, carbonate-45 and bicarbonate-45 respectively by deconvolution data provided in Table 5.1. Having more Silk-II content in as cast films through formic acid is in good agreement with the literature, where they observed higher Silk-II content of 34.3% compared to films obtained from water (23.7%) and trifluoroacetic acid (26.9%)

[215]. Their degumming process was based on sodium carbonate and their results are comparable to ours.

Table 5.1. Different silk conformations content of films (*Before methanol treatment, **After methanol treatment)

Sample	β -sheet		Random coil		α -helix		β -turn		Side chains and others	
	Before*	After**	Before	After	Before	After	Before	After	Before	After
Ammonia-45	46.2	49.5	12.7	12.2	6.2	4.2	15.2	15.2	19.6	18.9
Ammonia-60	31.8	43.0	16.5	11.9	1.1	10.0	23.8	16.2	26.8	18.9
Ammonia-90	31.8	43.9	16.4	9.8	1.2	10.4	21.5	16.1	29.0	19.8
Carbonate-45	33.9	42.4	11.8	16.1	2.5	10.9	22.3	14.6	29.5	16.0
Bicarbonate-45	35.9	42.4	13.1	3.9	0.8	4.1	21.7	23.8	28.5	25.8

We can also see from the data provided in Table 5.1., that when fibers were degummed for prolonged time, ammonia-60 and ammonia-90, Silk-II content decreased drastically from 46.2% to 31.8%. These results show that, if degumming is performed under mild conditions like ammonia-45 in our case, where less degradation is involved, as cast films have much higher content of Silk-II. This higher content of Silk-II (β -sheet) and Silk-I (α -helix) can be obtained without any further treatment with water or methanol if degumming is performed under mild conditions. All the films were further treated with methanol/water (4/1) mixture to see the impact on conformational changes in SF. Extracted deconvolution data shows that more changes occurred in films where less Silk-II content was present in as cast films. In all films other than ammonia-45, Silk-II content reached to 42-44%. However, it is still less than as cast film of ammonia-45. Also, considerable increase in α -helix was observed after methanol/water mixture treatment. This is most probably due to the water present in treatment mixture and skin like layer formation as discussed before. In ammonia-45, Silk-II content enhanced to ~50% after treatment. So conclusively, we can say that films cast from formic acid overall produce more β -sheet or Silk-II content. Furthermore, degumming conditions also impact crystallinity of the films produced. Severe degradation involved in harsher chemical treatments like carbonate and bicarbonate results in enhanced amorphous content.

5.3.2.2 *X-ray diffraction analysis*

X-ray diffractograms for cocoons and regenerated silk fibroin films before and after methanol/water mixture treatment are reproduced in Figure 5.6. Diffraction peaks corresponding to Silk-I and Silk-II are shown in red and black arrowheads respectively. In XRD profiles of the films obtained from degummed fibers by ammonia process, a small, but sharp peak at 12.2° , which corresponds to Silk-I conformation is observed. This peak is absent in films obtained using carbonate and bicarbonate processes. This implies that ammonia processed films have distinct α -helical domains than others. The distinct nature of hard and soft domains is discussed in AFM analysis in Figure 5.7. In Ammonia-45 sample there are two extra peaks at 41.3° and 44.8° (Figure 5.6-a), which shift slightly to 40.3° and 44.3° respectively, after methanol treatment (Figure 5.6-b). These peaks, which are observable in ammonia-based films only, can be assigned to Silk-I and are also confirmed by FTIR analysis. These peaks sustain even after methanol/water mixture treatment due to the presence of water and giving rise to Silk-I configuration. On the other hand, severe degradation is observed in carbonate and bicarbonate-based films representing typical diffraction patterns of amorphous silk. Figure 5.6-b shows XRD patterns obtained for films after methanol/water (4/1) mixture treatment. Silk fibroin macromolecules differ in size considerably in organic and aqueous media due to different molecular conformations. Adding solvents like alcohols starts β -folded conformation and converts water soluble silk-I to water insoluble silk-II [79, 216]. After methanol treatment, the peak at 12.2° in ammonia-based film disappears, while two new peaks at 40.3° and 44.3° are observed due to formation of a different Silk-I conformation. In as cast films, Silk-I configuration appeared at 12.2° in the absence of water. In the presence of water, it changed its form to another preferential configuration of Silk-I. By summing up FTIR and XRD analysis of films, we can say that ammonia-based films possess more Silk-II content before and after methanol treatment than other films obtained through carbonate and bicarbonate degumming. Before methanol treatment, carbonate and bicarbonate films have more amorphous structure, which is partially converted to silk-II after methanol/water treatment as discussed in FTIR analysis.

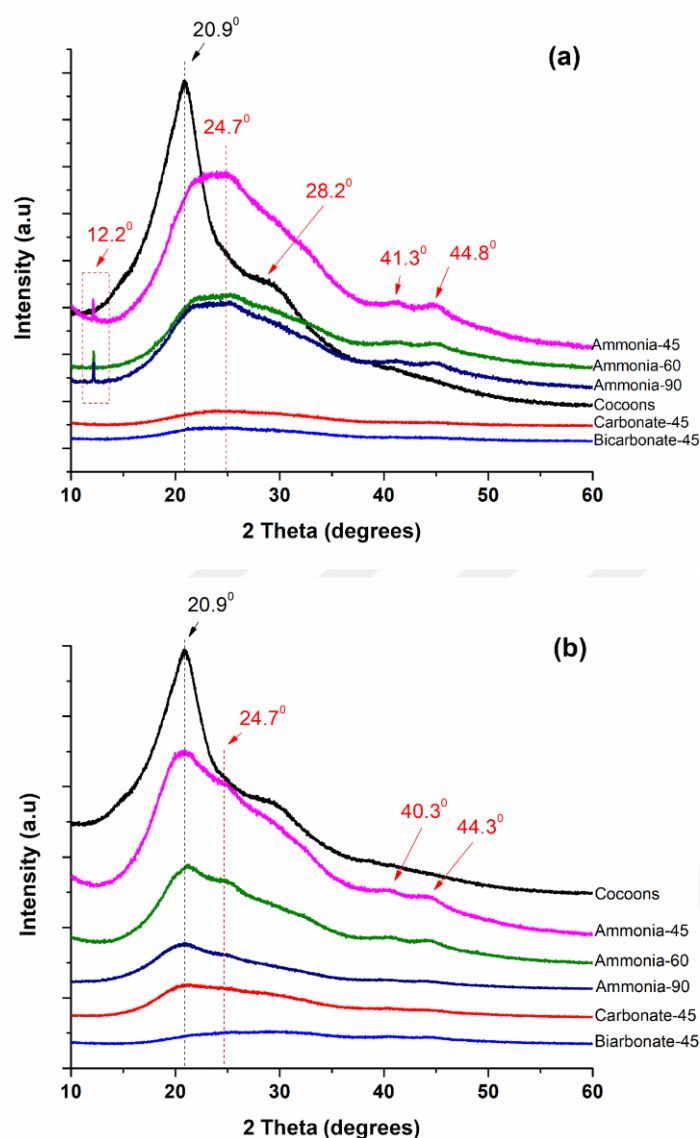


Figure 5.6. XRD diffractograms of cocoons and regenerated silk fibroin film: (a) before (b) after methanol treatment.

Therefore, silk fibroin films produced by using ammonia based degumming process can be good candidates for having balanced Silk-I and Silk-II structures to provide the required properties for biomaterials. TGA analysis of silk fibroin films developed through different reagent and processing conditions was also performed. Thermal degradation of these films seems to be somewhat dependent on reagent type and processing conditions. Ammonia based films showed delayed thermal degradation in 500-700°C (TGA data for all films before and after methanol/water mixture treatment is provided as Appendix-VI)

5.3.2.3 AFM analysis

For AFM analysis, 10% w/w solution of regenerated silk fibroin for each sample was prepared in formic acid and spin coated on glass slide at 1000 rpm for 70 sec. Spin coated glass slides before and after methanol/water mixture treatment were dried under vacuum at ambient temperature and analyzed. AFM images obtained are reproduced in Figure 5.7(a), (b) and (c). It is important to note that AFM is a surface sensitive method and the results provided here describe the surface morphologies, but not necessarily the bulk. Phase images before methanol treatment (a), after methanol treatment (b) and amplitude error images after methanol treatment (c) are obtained. Figures 5.7(a) show that α -helical and amorphous silk fibroin structure is present in all samples. This presence is more distinct and noticeable in film originated from ammonia based process. Ammonia-45 based films possess more distinct phase separated hard (bright regions) and soft (dark regions) domains. Phase images obtained after washing with methanol/water mixture in Figures 5.7(b), show almost mixed phase obtained in all samples with reduced phase separation in ammonia based films as well due to decrease in the random coil and α -helical structures as predicted by peak resolved data. For better understanding crystalline domains in treated films, amplitude error images were obtained as represented in Figures 5.7(c).

(a)

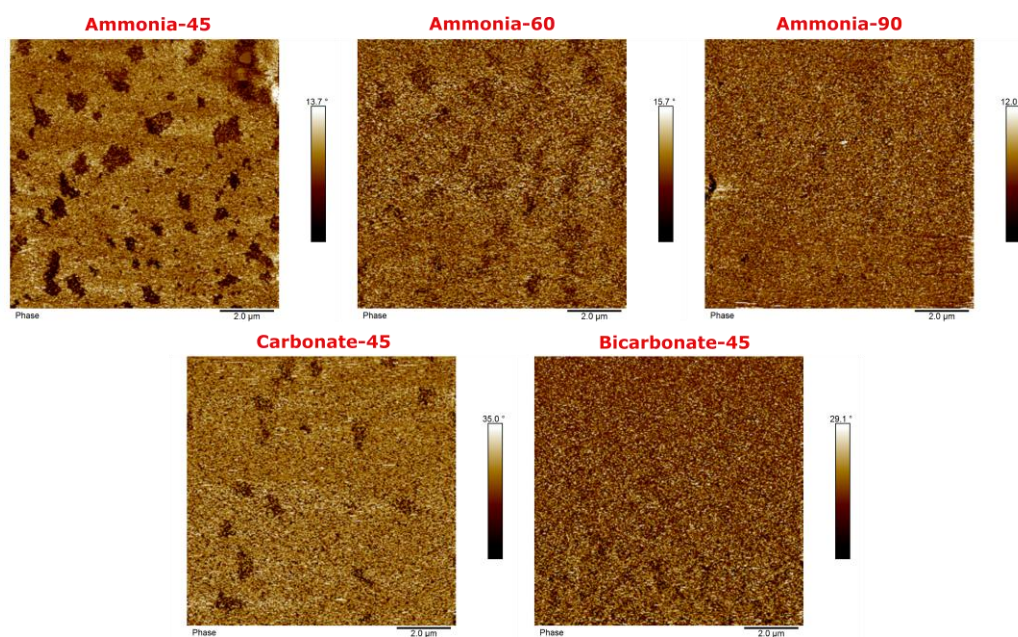


Figure 5.7(a). Phase images of SF films obtained through different processes before methanol/water treatment (Scale bar=2 μ m)

Amplitude error image analysis is a better alternate to recognize crystalline domains on substrate surface rather than phase, height and topographical analysis in AFM [217]. Quite interesting results were observed in amplitude error images of silk fibroin films. We can clearly observe ordered domains of silk fibroin at nano level in terms of micro/nano phase separation. Size and number of these ordered molecular domains is dependent on the processing conditions of silk fibroin.

(b)

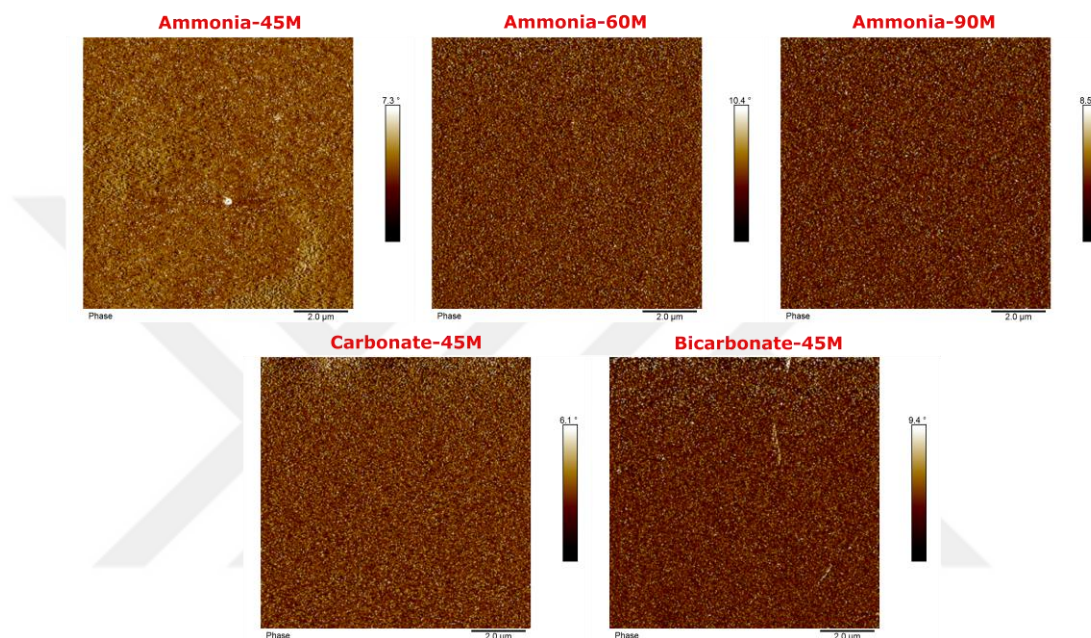


Figure 5.7(b). Phase images of SF films obtained through different processes before methanol/water treatment (Scale bar=2μm)

AFM results in conjunction with FTIR and XRD show that even cocoons degumming process and conditions influence the ultimate properties of material. The ordered domains observed on silk fibroin films is dependent on type of reagent used in process and processing time as well. Larger and more in number distinct crystalline domains were observed in case of ammonia-45 based film. Other reagents (carbonate-45 and bicarbonate-45) and prolonged treatment (ammonia-60 and ammonia-90) result in higher amount of amorphous regions and less amount of crystalline domains. Hence, these amplitude error images can be used to identify crystalline domains in polymeric films where phase and topographical images couldn't serve the purpose.

(c)

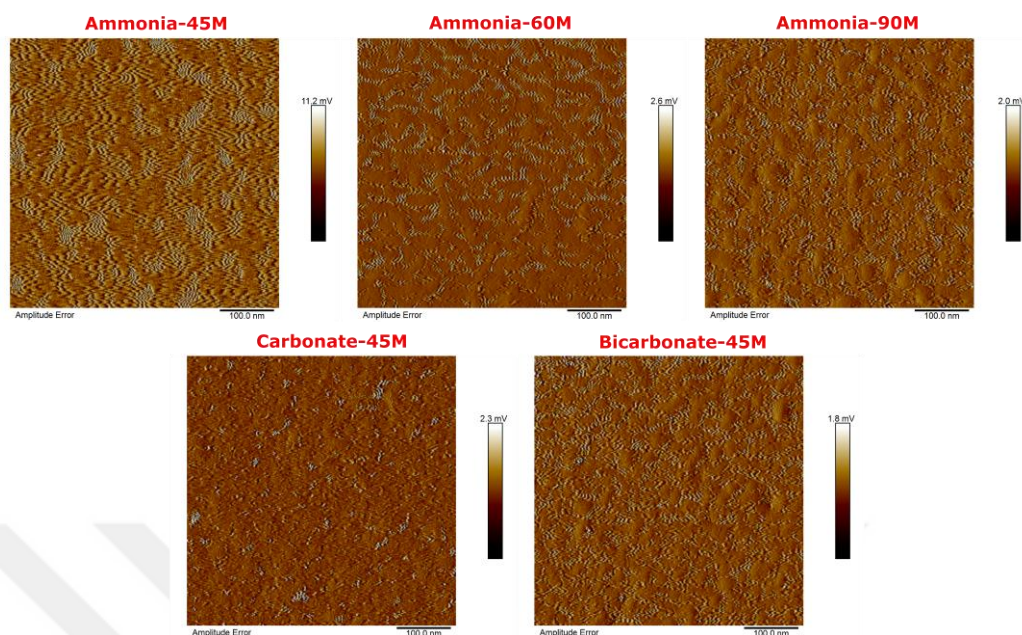


Figure 5.7(c). Phase images of SF films obtained through different processes before methanol/water treatment (Scale bar=100nm)

5.3.2.4 Water contact angle measurement

Because of the varying water-soluble Silk-I, insoluble Silk-II structures and micro/nano-phase separations present in sample surfaces, as determined by AFM studies, water contact angle measurement was performed to check surface hydrophilicity. 10 μ L droplet of double distilled water was used for static contact angle measurements. Figure 5.8 shows the contact angles and respective images for all samples. We can clearly see that for silk fibroin film developed through ammonia solution-based degumming process is more hydrophilic than others (37° for ammonia-45 and about 60 to 62° for carbonate and bicarbonate-45). When the treatment time is increased for ammonia-based films like ammonia-60 and ammonia-90, hydrophilicity is decreased ($\sim 55^\circ$). When films were treated with methanol/water mixture, contact angles increased for all samples as expected due to the conversion of silk-I to silk-II [92]. Increase in water contact angle after treatment is in accordance with the results obtained and discussed in Figures 5.5, 5.6 and 5.7. Increase in contact angle is more evident in case of ammonia films due to the higher phase separation between Silk-I and Silk-II domains.

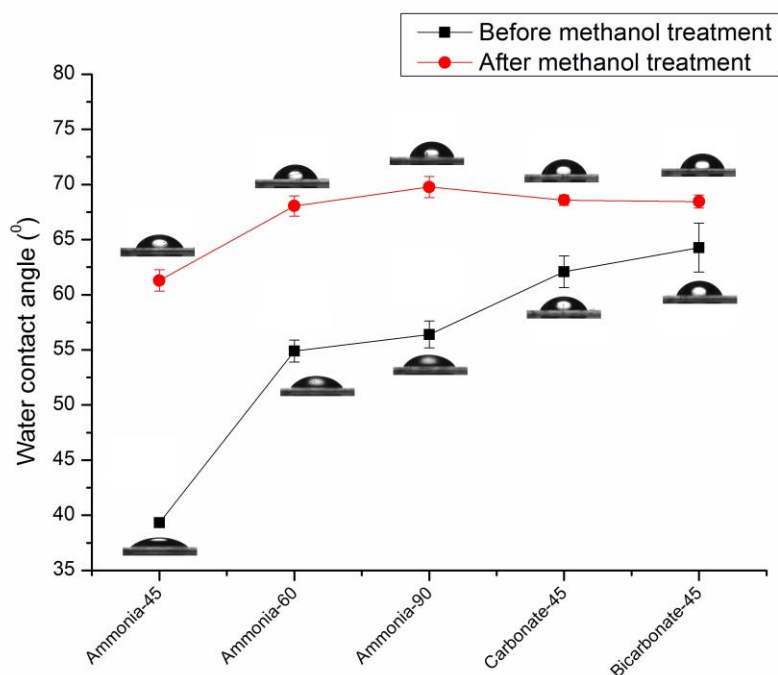


Figure 5.8. Water contact angle on silk fibroin films before and after methanol treatment

The most interesting observation of this study is that even though more β -sheet content is observed after methanol treatment of ammonia processed sample, it is slightly more hydrophilic than others due to nano phase separation of water soluble and insoluble domains thus providing more possibility of water molecules attachment as compared to mixed phases. These water stable films due to adequate crystallinity and being more hydrophilic at the same time is quite important and useful for cell attachment and controlled degradation [210].

5.4 Conclusions

In this study, silk (*Bombyx mori*) cocoons have been degummed using 0.3 w/w% solutions of sodium carbonate (45 min), sodium bicarbonate (45 min) and ammonia (28-30%) solution (45, 60 and 90 min). Degummed fibers were dissolved in phosphoric acid/formic acid (7:3 v/v), coagulated in methanol, washed, and dried to obtain pure silk fibroin powders. The powder was re-dissolved in formic acid and films were cast. Degummed fibers and films were characterized through various techniques. Effects of degumming reagent and time of processing were critically analyzed. This novel degumming of cocoons

through ammonia solution and dialysis free process for silk fibroin material fabrication was found to be quite interesting in terms of chemical structure and physical properties. Films obtained from ammonia solution (45 min) produce material that have suitable content of crystalline structure and possess high hydrophilicity due to nano phase separation of water soluble and insoluble domains. We believe that these materials can be useful for blending with other polymers to acquire optimum mechanical properties along with better cell attachment, proliferation, cytocompatibility, controlled degradation for tissue engineering applications.



CHAPTER 6

FABRICATION OF PCL/SF NANO-FIBROUS SCAFFOLDS

6.1 Summary

Fabrication of polycaprolactone/silk fibroin (PCL/SF) nano-fibrous composite scaffolds through novel processes is reported. PCL was synthesized through ring opening polymerization of ϵ -caprolactone using 1,2-bis(3-aminopropoxy)ethane as initiator. Reactions were carried out in xylene solution at $130\pm 5^\circ\text{C}$ under the catalysis of tin octoate. As already described, *Bombyx mori* cocoons were degummed using 0.3% w/w solutions of boiling ammonia (28-30%) for 45 minutes. Washed and dried degummed fibers were dissolved in 7/3 volume mixture of phosphoric acid and formic, coagulated in methanol and filtered. After washing, silk fibroin (SF) was used for subsequent processes. Electrospinning technique was used to fabricate nano-fibrous PCL/SF scaffolds. PCL solutions containing different amounts of SF (0, 20 and 40 w/w%) were prepared in formic acid. Electrospinning of PCL and composite solutions was carried out at appropriate processing conditions. Nano-fibrous scaffolds obtained were purified by washing and drying and were characterized by scanning electron microscopy (SEM), attenuated total reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy, X-rays diffraction (XRD) and water contact angle measurements. Cell viability analysis was performed by seeding Human BJ fibroblast cells. This *in vitro* analysis shows that these scaffolds are non-toxic and incorporation of SF resulted in enhanced proliferation. So, these nano-fibrous scaffolds developed through dialysis free processing of silk fibroin can be promising substrates used for skin tissue engineering applications.

6.2 Materials and Methods

6.2.1 Materials

Silk (*Bombyx mori*) cocoons were kindly supplied by Idealab, Koc University, Istanbul. Ammonia solution (28-30%), THF, phosphoric acid, formic acid (98%), reagent grade xylene (mixture of isomers) and ϵ -caprolactone were purchased from Aldrich and were used as received without further purification. Technical grade methanol was used for coagulation. 1,2-Bis(3-aminopropoxy)ethane (Jeffamine[®] EDR-176 (XTJ-590)) was obtained from Huntsman. Stannous octoate (T-9) was obtained from Air Products. L-glutamine (200 mM), trypsin-EDTA (25 %), were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, phosphate buffer saline (PBS) were purchased from Gibco (Grand Island, NY). CellTiter-Glo luminescent cell viability assay was purchased from Promega (Madison, WI, USA). Silk cocoons were degummed through ammonia solution and fibers were further processed to obtain SF. Complete procedure is already well explained in Chapter 5, sections 5.2.2.1 and 5.2.2.2.

6.2.2 Methods

6.2.2.1 PCL synthesis through ring-opening polymerization

Polycaprolactone was synthesized by the ring-opening polymerization of ϵ -caprolactone (CL). Proposed reaction scheme is presented in Figure 6.1. Difunctional amine (EDR 176) was used as an initiator together with T-9, which was the catalyst. Molecular weight of PCL was controlled by the ratio of CL to EDR 176. Synthesis was carried out according to our previously published method [144] with minor modifications. Reactions were carried out in 500 mL, three neck round bottom Pyrex flasks equipped with overhead stirrer and thermometer.

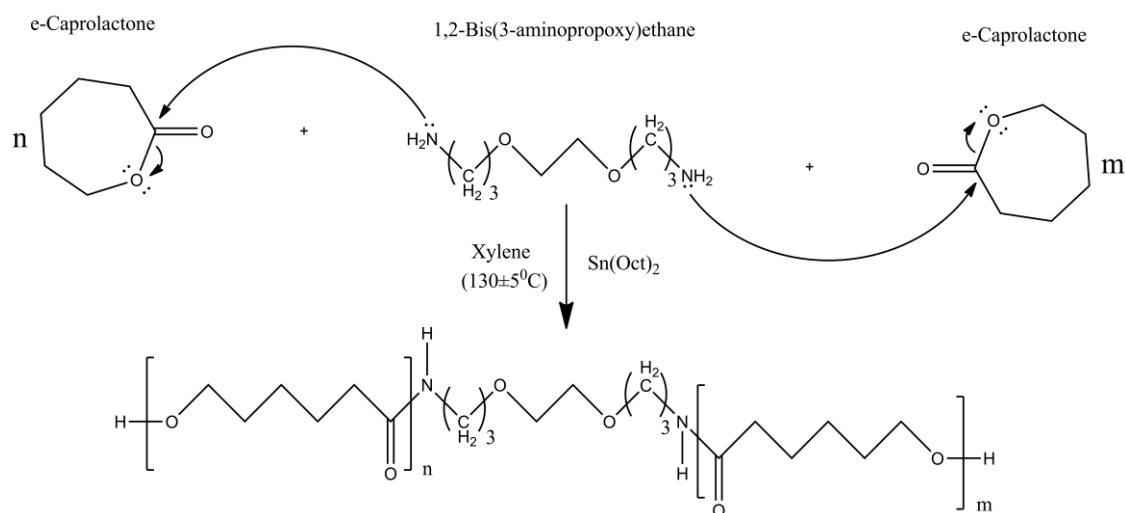


Figure 6.1. Schematic representation of PCL synthesis through ROP

Typical procedure followed for high molecular weight PCL synthesis was as follows: 30 g of CL, 0.049 g of initiator and 0.104 g of T-9 catalyst were taken into the reaction flask. Heating was started, while the mixture was stirred at 120 RPM. Temperature was maintained at $130\pm 5^{\circ}\text{C}$ throughout the reaction. Xylene was added slowly to maintain viscosity of the reaction mixture. Reaction was completed in about 24 h and system was cooled down to room temperature. THF was added to dilute the system to obtain polymeric solution about 20 w/w% solids. Polymer was coagulated in methanol (polymer solution/methanol in 1/10 ratio) under strong agitation. Residue was filtered and dried in air oven at 50°C until constant weight. Synthesized PCL ($M_w=85$ kDa, $M_n=43$ kDa by GPC) was used for fabrication of PCL/SF composite scaffolds through electrospinning.

6.2.2.2 PCL/SF solutions and their electrospinning

Nano-fibrous scaffolds were developed through “green electrospinning process”. Less toxic Q3C class-3 solvent, formic acid, was used instead of commonly used class-2 toxic solvents like THF and DMF [218]. First, calculated amount of SF was dissolved in formic acid. Then desired amount of PCL was added and the stirred for 2 h. Solutions obtained were directly used for fabrication of scaffolds through electrospinning. After electrospinning, scaffolds were peeled off from aluminum foil by soaking in methanol and left in fume hood overnight. Then, scaffolds were treated with ammonia solution for 1h to

neutralize the formic acid residue, followed by frequent washing with distilled water. After drying, scaffolds were again treated with methanol for 1 h to induce crystallinity in silk fibroin. Then scaffolds were dried in vacuum oven at RT until constant weight. Dried scaffolds were characterized and used in subsequent processes. Concentration and optimized conditions of electrospinning for each solution is presented in Table 6.1.

Table 6.1. PCL/SF concentrations, electrospinning conditions and average fiber diameter

Sample code	(20 w/w%) Solutions in FA		Voltage (kV)	Distance (cm)	Feed rate (μ L/min)	Average fiber diameter (nm) n=10
	PCL%	SF%				
PCL	100	0	17.5	12	5	151 $\pm$ 38
PCL/SF-20	80	20	17.5	12	5	196 $\pm$ 53
PCL/SF-40	60	40	17.5	12	5	284 $\pm$ 115

6.2.2.3 Characterization techniques

ATR-FTIR of samples were obtained on Thermo-scientific iS50 FTIR spectrometer having ATR attachment. Absorption spectra for all samples were obtained at autogain, resolution of 2 cm^{-1} and 64 scans. Surface analysis was done on Zeiss EVO-LS-15 SEM. Prior to analysis; samples were sputter coated with 10 nm layer of gold to make surface conductive. Contact angle measurements were made on dataphysics OCA 35 by using double distilled water (10 μ L droplet for each measurement). Gel permeation chromatography (GPC) was performed on a Viscotek VE 2001 series instrument equipped with Dguard, D2500, D4000 and D5000 columns and refractive index detector (VE3580). Mobile phase used was THF at 35 $^{\circ}\text{C}$, at a flow rate of 1mL/min. Synthesized PCL was dissolved in THF at a concentration of 1 gm/mL. Molecular weight was determined by using polystyrene standards calibration curves. Stress-strain analysis of developed scaffolds was performed on Instron Model 4411 universal testing machine at room temperature with crosshead speed of 25 mm/min. Samples were cut with a dog-bone shaped die (ASTM D1708). The initial length of samples was 24 mm.

6.3 Results and discussions

6.3.1 ATR-FTIR analysis

ATR-FTIR analysis was performed on the scaffolds to demonstrate SF incorporation. Spectra for extracted SF powder from degummed fibers was also obtained to compare the interactions between PCL and SF in composite fibers.

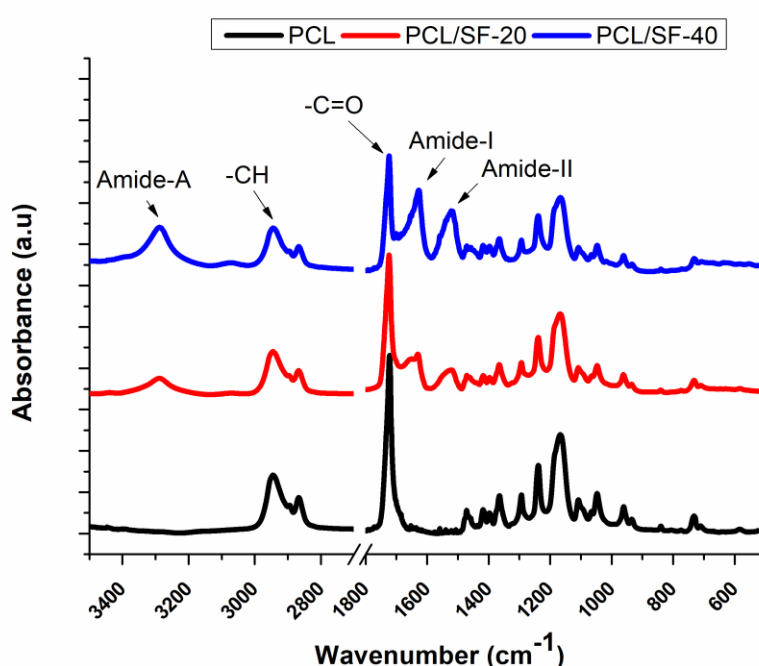


Figure 6.2. ATR-FTIR analysis of PCL and PCL/SF composite scaffolds having different ratios of SF

For a better comparison, only 3500-2700 cm^{-1} and 1800-1000 cm^{-1} FTIR regions are shown in Figure 6.2. Major bands in Amide-A, Amide-I ($\text{C}=\text{O}$ stretching) and Amide-II ($\text{C}-\text{N}$ stretching and $\text{N}-\text{H}$ in plane bending vibrations) regions for SF powder were present at 3278, 1618 and 1513 cm^{-1} respectively. Very sharp and symmetrical H-bonded $\text{N}-\text{H}$ absorption band at 3278 cm^{-1} present in SF powder was slightly shifted to 3290 cm^{-1} in PCL/SF composites. Similarly, a small shift was also detected in Amide-I and Amide-II regions and new bands were observed at 1628 and 1521 cm^{-1} . This shows good compatibility in these composites through strong interactions between PCL and SF.

Characteristic and fairly strong C=O stretching band for PCL was present at 1722 cm^{-1} in pure PCL and PCL/SF composites. The presence of SF Amide bands along with PCL characteristics bands, CH₂ stretching (2943 and 2865 cm^{-1}), C=O stretching (1722 cm^{-1}) and C-O stretching (1293 cm^{-1}) confirms the successful incorporation of SF in PCL matrix in electro-spun composite scaffolds. These results are also in accordance with the literature [219, 220].

6.3.2 Surface analysis

Surface morphology of nanofibers in scaffolds was examined through SEM. Samples were pre-coated with 10 nm layer of gold to make surface fairly conductive.

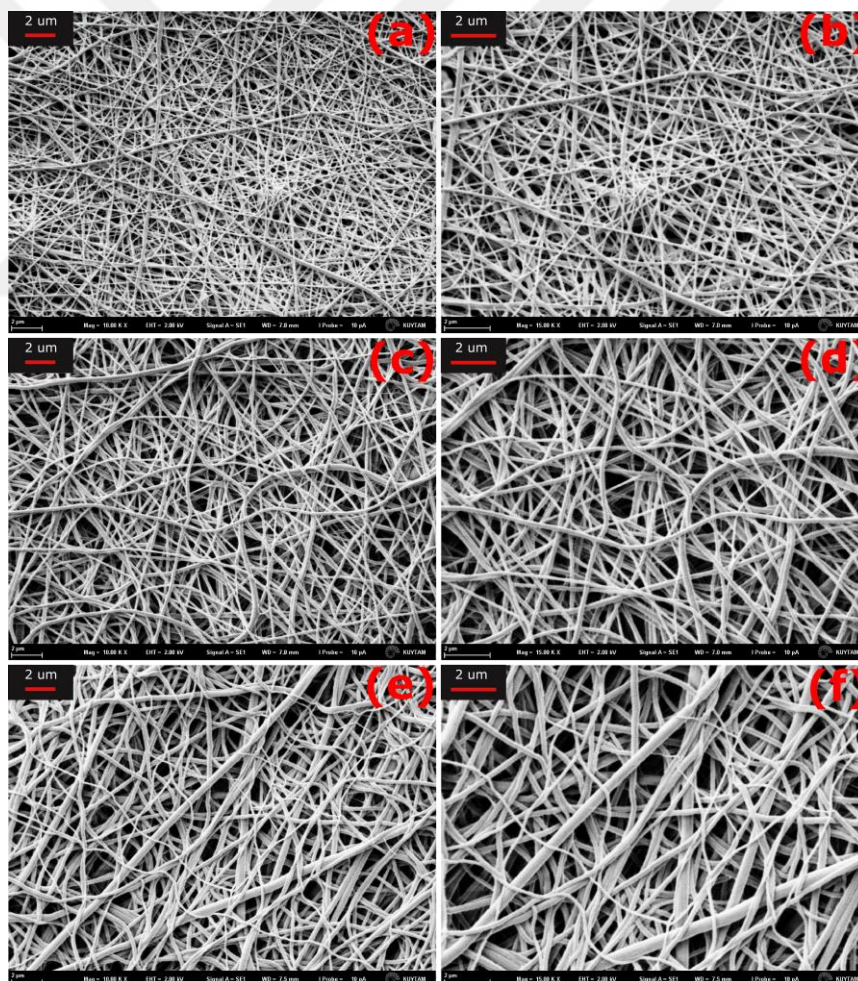


Figure 6.3. SEM analysis of PCL (a, d) and PCL/SF-20 (c, d) and PCL/SF-40 (e, f) nanofibrous scaffolds.

Fiber diameter was measured using ImageJ[®] software. Average of 10 values along-with SD is provided in Table 6.1. Wetting behavior of composite scaffolds were also investigated. For comparison purposes, same solutions were also spin coated on glass slides with spin coater at 1000 rpm and for 70 sec. Contact angle measurements along with images are provided in Figure 6.4.

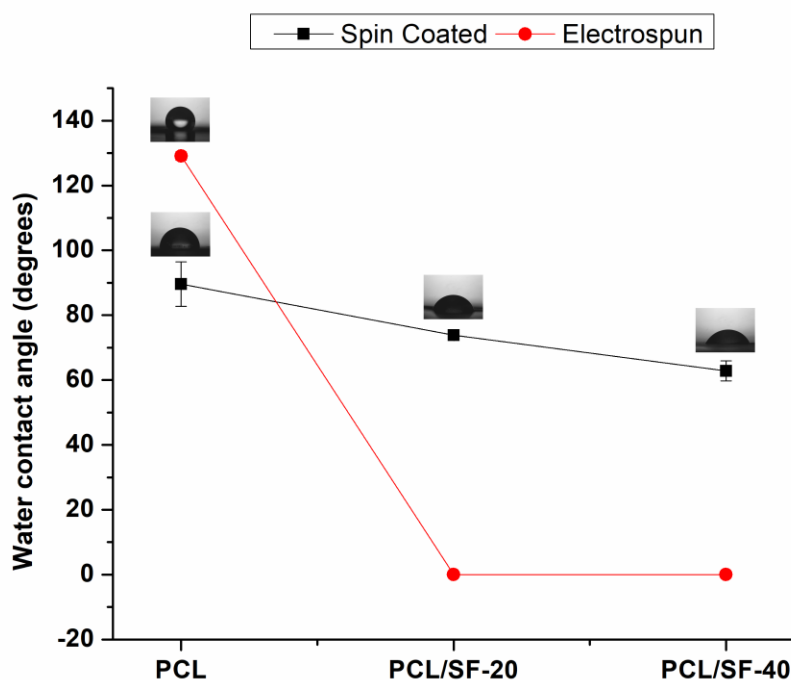


Figure 6.4. Water contact angle measurements for spin coated and electro-spun scaffolds

CA for PCL spin coated sample was $89.6 \pm 6.9^\circ$ that was lowered down considerably to $73.8 \pm 1^\circ$ and $62.8 \pm 3^\circ$ after 20% and 40% SF incorporation respectively. The results are as expected. Higher angle is due to the hydrophobic nature of PCL and surface was changed to hydrophilic due to SF incorporation in the system. Hydrophilicity of surface was further increased with increasing SF content%. Interesting results were observed in electro-spun scaffolds. CA for electro-spun PCL scaffold was observed as $129.1 \pm 1.3^\circ$. Electro-spun surface is quite hydrophobic than spin coated surface due to surface roughness present. However, after SF inclusion into the system, either 20% or 40% by weight, surface became super-hydrophilic showing almost 0° contact angle. This can be due to two reasons: hydrophilic nature of SF and capillary effect generated because of micro/nano pores

present on the surface. Water interacts to the hydrophilic groups present on the surface and is attracted towards the inside of the web very rapidly due to capillary effect. This combination of a hydrophilic surface and capillary effect (which may also be due to Wenzel type wetting) gives rise to a super-hydrophilic surface. These composite scaffold surfaces can be a good platform for cell attachment and growth.

6.3.3 Degradation studies

Degradation of nano-fibrous scaffolds of PCL and PCL/SF composites was monitored in DMEM medium having 10% FBS over 15-weeks period of time. Before incubation, scaffolds were pre-dried in vacuum oven at RT for 24 h to remove any water residues. After weighing, samples were incubated in DMEM+FBS solution at 37 °C. Samples were taken out from oven after 1, 3, 7, 11 and 15 weeks. Samples were first washed with distilled water and then surfaces were cleaned with warm water to remove any residues of incubation media. After drying in vacuum oven at RT until constant weight mass loss% was calculated by Equation 6.1.

$$\text{Weight loss (\%)} = \frac{W_t - W_i}{W_i} \times 100 \quad \text{Equation 6.1}$$

Where “Wi” and “Wt” denote the initial and final weight respectively. FBS containing DMEM medium was changed subsequently at each time interval of analysis. The results obtained after degradation analysis are provided in Figure 6.5. As expected, pristine PCL scaffolds exhibited almost negligible degradation over 15-weeks of incubation, while SF containing scaffolds showed considerable decrease in weight as compared to PCL. After 15 weeks, mass percent remained for PCL/SF-20 and PCL/SF-40 samples were 86.6±4.7% and 77.3±1.2% respectively. With the increase in SF content, mass loss was higher. Slow degradation of PCL and rapid degradation of SF can be a good combination for various applications. SF can be utilized for initial attachment of cells and their growth and degrade before PCL. In the meanwhile, natural ECM will be developed by cells and ultimate support provided by PCL will be slowly diminishing as it degrades.

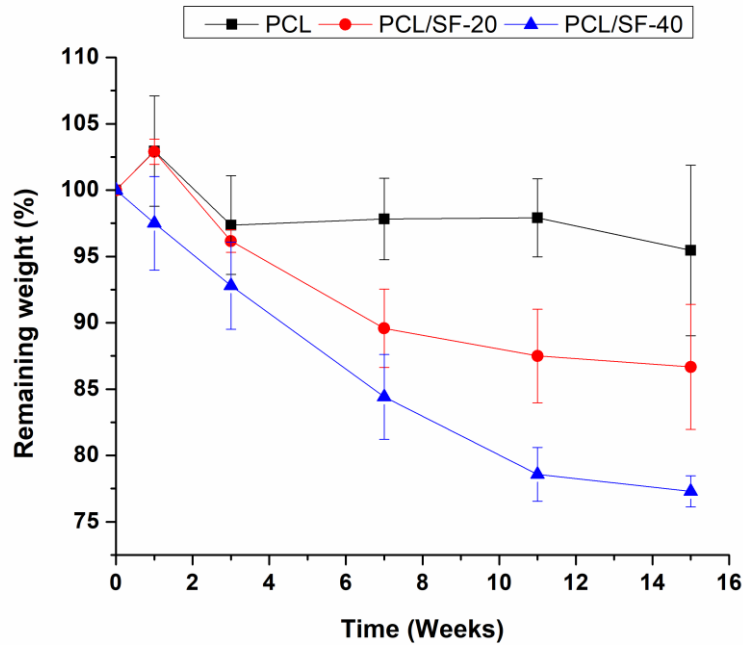


Figure 6.5. Degradation studies of PCL and PCL/SF nano-fibrous composite scaffolds

6.3.4 Mechanical properties of scaffolds

The ultimate tensile strength (UTS), elongation at break and elastic modulus (EM) values for all scaffolds were measured and compared with the natural skin (Human skin) from literature [221]. Results are provided in Figure 6.6. There is no significant change observed in mean UTS values of PCL and PCL/SF composites. However, elongation at break was reduced significantly from $42.2 \pm 2.4\%$ (PCL) to $21.1 \pm 0.4\%$ (PCL/SF-40). Elastic modulus significantly increased after SF incorporation from 21.6 ± 1.7 MPa (PCL) to 49.3 ± 6 MPa (PCL/SF-20) and 98.1 ± 23.7 MPa (PCL/SF-40) as expected. If we compare UTS, elongation and EM of human skin, then we can say PCL/SF-40 composite scaffold can be somehow compared and can be a better skin substitute.

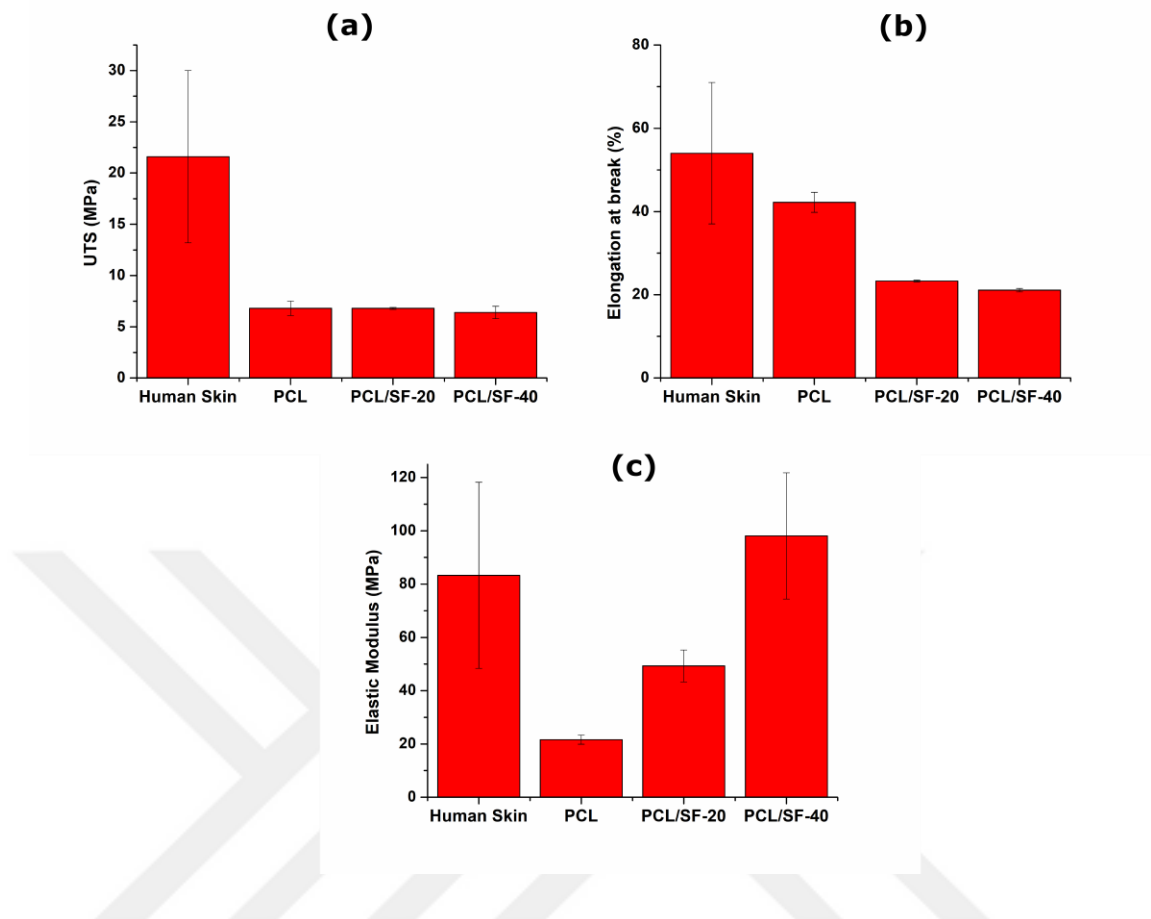


Figure 6.6. Mechanical properties comparison of PCL, PCL/SF and human skin: a) ultimate tensile strength, b) elongation at break and c) elastic modulus

6.3.5 *In vitro* cell viability analysis

Cytotoxicity of PCL and PCL/SF composite scaffolds were determined using adenosine triphosphate (ATP) dependent cell viability assay, CellTiter-Glo (Promega), as per manufacturer's description. Human BJ fibroblast cells (Passage # 8–16) were cultured with DMEM supplemented with 10 % FBS, 3.97 mM L-glutamine, and 1% Penicillin/streptomycin in an incubator with 5% CO₂ at 37 °C. Scaffolds were washed with PBS and after drying, they were placed in 24-well plate and sterilized under UV light for 30 minutes. The samples were rinsed in DMEM at 37 °C and medium was changed 2 times before cell seeding. Fibroblast cells were seeded onto the scaffold's surface (15000 cells/0.2 cm²).

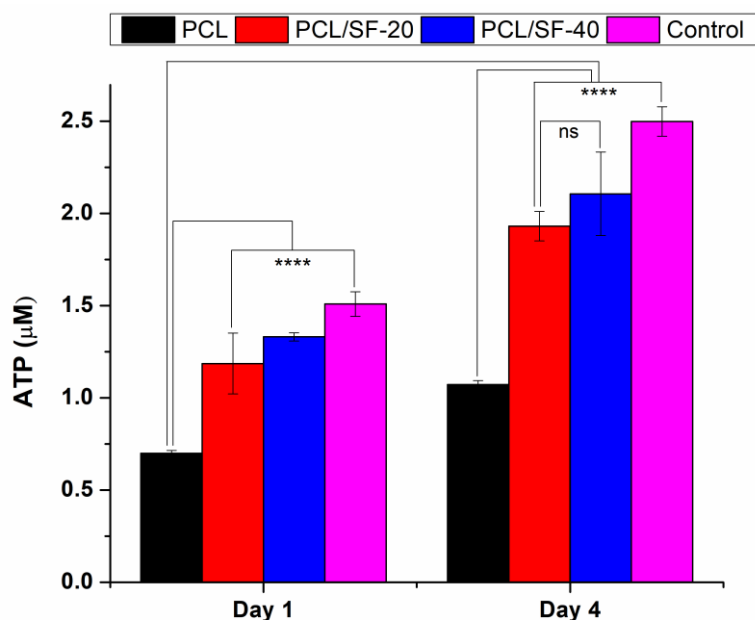


Figure 6.7. ATP release in cell seeding analysis measured through luminescence (n=5)

ATP standard curve was prepared in DMEM, prior to measurement. Both samples and ATP solutions were treated with CellTiter-Glo reagent and incubated at 25 °C, 100 rpm for 15 min. Luminescence values were measured on day 1 and day 4, using a plate reader (Biotek, Synergy H1). CellTiter-Glo reagent enables the luciferase reaction in catalytic presence of ATP. This reaction yields inactive oxyluciferin, adenosine monophosphate, CO₂, and light. This released light is measured in plate reader. After cell lysis, ATP is released from viable cells only, so luminescence value can be directly correlated with the number of viable cells present [222]. Two-way ANOVA followed by Tukey's multiple comparison test was performed to evaluate results. As we can see from Figure 6.7, Day-4 viability is significantly higher than Day-1 for all samples (****p<0.0001). Similarly, on Day-1 and Day-4, significant number of cells were present on composite scaffolds as compared to pristine PCL (****p<0.0001). It shows that these scaffolds are not toxic to fibroblast cells. Further, by incorporation of SF, cells proliferation rate was increased in PCL/SF composites than PCL scaffolds.

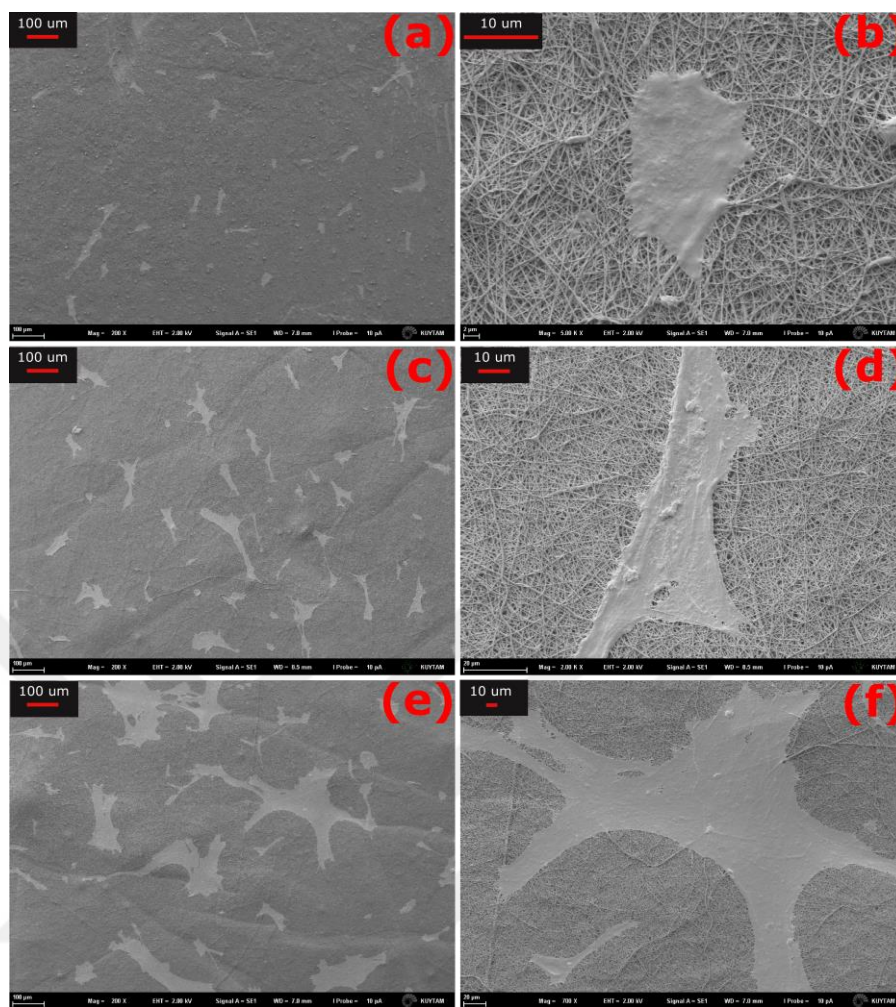


Figure 6.8. Surface morphology of cells on scaffolds: PCL (a, b); PCL/SF-20 (c, d) and PCL/SF-40 (e, f).

After 4-days of incubation, cells on scaffold surfaces were fixed with 2.5 % glutaraldehyde solution, followed by dehydration with increasing concentration gradient of ethanol in water (30%, 50%, 70%, 90% and 100% for 5 minutes each). Scaffolds were dried at ambient conditions first and then in vacuum oven at room temperature. Cell containing samples were mounted on double sided carbon tape attached stubs and coated with 15 nm gold layer through sputter coating. Morphology of the cells on scaffolds was examined through SEM and shown in Figure 6.8. Cells present on all scaffolds can be seen. Wide spreading was observed in case of SF incorporation because of more cells present on the surfaces. When cell membranes were collapsed due to ethanol dehydration, then these cells were accumulated to each other causing larger area spreading.

6.4 Conclusions

Fabrication of PCL and PCL/SF nano-fibrous scaffolds through electrospinning for use in skin tissue engineering applications is reported. PCL was synthesized through ROP of ϵ -caprolactone using 1,2-Bis(3-aminopropoxy)ethane oligomer as initiator. SF was obtained through novel degumming process and influence of regenerated SF incorporation to PCL was evaluated on biomechanical properties of the scaffolds. SEM analysis showed nano-fibrous scaffold development. Successful incorporation of SF in composite scaffolds was confirmed by ATR-FTIR analysis. Surface properties were changed from hydrophobic (110° contact angle) to super-hydrophilic after SF incorporation. Scaffolds developed were analyzed through cell seeding as well. For this purpose, Human BJ Fibroblast cells were used. ATP assay showed that cell proliferation rate was enhanced significantly in PCL/SF composite scaffolds. Mechanical analysis of these scaffolds revealed that scaffolds possess enough tensile strength and elasticity to be used as skin grafts even in PCL/SF composite scaffolds. So conclusively, we can say that these PCL/SF nano-fibrous composite scaffolds developed through dialysis free processing of silk fibroin can be promising substrates used for skin tissue engineering applications.

CHAPTER 7

FABRICATION OF PU/SF NANO-FIBROUS SCAFFOLDS

7.1 Summary

Development of novel degradable polyurethane/silk fibroin (PU/SF) nano-fibrous scaffolds for skin tissue engineering applications is reported. Polyurethane was synthesized by the stoichiometric reaction of hydroxyl terminated PCL oligomer ($M_n=2000$ g/mol) (PCL2k) and 1,6-hexamethylene diisocyanate (HDI) under the catalysis of dibutyltin dilaurate (DBTDL). Silk (*Bombyx mori*) cocoons were degummed and processed to obtain silk fibroin (SF). 15% w/w PU solutions in formic acid were prepared and nanocomposites containing 10 and 20 w/w% SF were prepared. Nano-fibrous scaffolds were successfully prepared by electrospinning using these solutions. Scaffolds obtained were characterized by various techniques, such as; scanning electron microscopy (SEM), infrared spectroscopy (ATR-FTIR), x-ray diffraction (XRD), stress-strain analysis and contact angle measurements. Nano-fibrous structure, successful incorporation of SF, retention of crystalline nature of PU in formic acid, mechanical performance and surface modification of these scaffolds were verified through characterization studies. NIH-3T3-mouse embryo fibroblast cells were seeded on these scaffolds. Proliferation of fibroblast cells after SF incorporation was enhanced almost 9 folds as compared to pure PU. We believe these scaffolds have enough mechanical and biological performance to serve as skin substitutes.

7.2 Materials and methods

7.2.1 Materials

Silk (*Bombyx mori*) cocoons were kindly supplied by Idealab, Koç University, Istanbul. PCL-diol ($M_n=2000$ g/mol) and HDI were received from Acros Organics. DBTDL was obtained from Witco and was used as 1 w/w% in THF. Ammonia solution (28-30%), formic acid, phosphoric acid, THF, methanol, and formic acid (98%) were received from Merck and were used as received. Silk cocoons were degummed through ammonia solution based degumming process. Degummed fibers were processed to obtain SF powder according to the procedure explained in Chapter 5, sections 5.2.2.1 and 5.2.2.2.

7.2.2 Methods

7.2.2.1 Polyurethane synthesis

PCL2k/HDI (1/1 by mol.) was synthesized according to our previously published method [223]. For the synthesis 20 grams of PCL2k, 1.682 grams of HDI and 32 grams of THF were introduced in a 2-neck round bottom flask equipped with an overhead stirrer and thermometer. System was heated to reflux. After complete dissolution of PCL, 100-150 ppm of T-12 catalyst (1 w/w% solution in THF) was added. Reaction was continuously monitored by FTIR spectroscopy by following the disappearance of typical –NCO peak at 2260 cm^{-1} [141]. After almost 2 h of reaction, -NCO peak disappeared. 0.15 grams of HDI diluted in THF was added further to cover up the loss of HDI due to possible side reactions. Reaction was further continued and time to time, THF was being added due to its evaporation to keep system diluted. Reaction scheme is presented in Figure 7.1. Second step of the reaction was completed in ~1 h as indicated by FTIR through complete consumption of –NCO groups. Solid weight% of polymer solution was ~20%. After cooling down to RT, polymer was coagulated in methanol/water (1:1) mixture in order to remove any unreacted monomers.

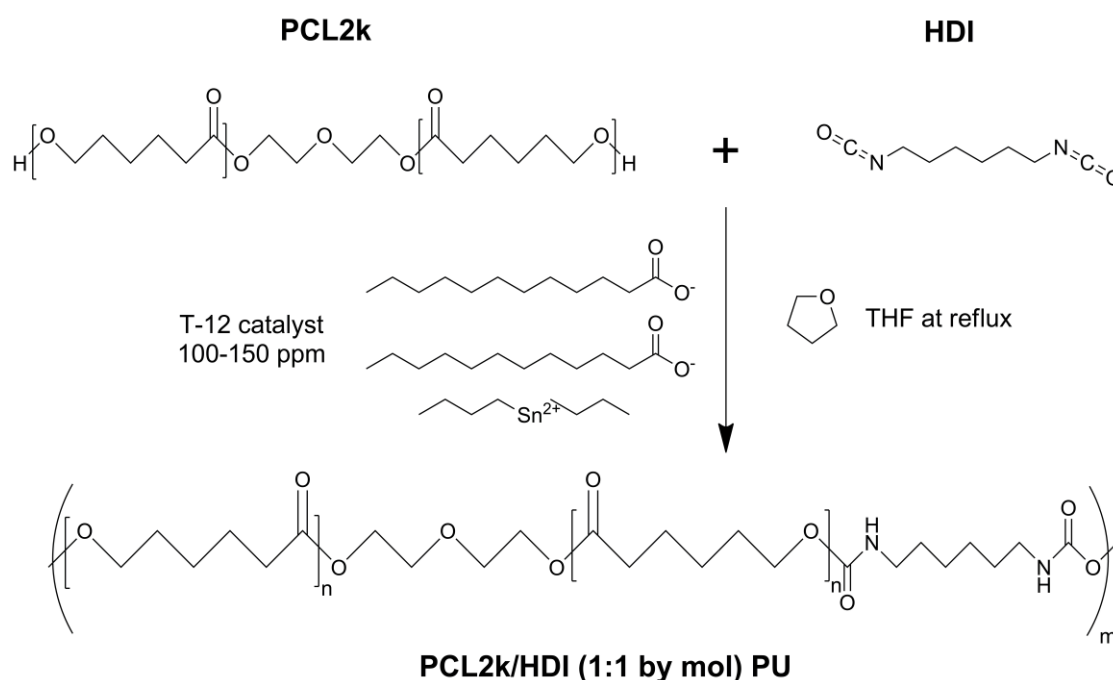


Figure 7.1. Schematic representation of PCL2k/HDI (1:1) polyurethane synthesis

7.2.2.2 Polyurethane/silk fibroin nano-fibrous scaffolds development

Synthesized PU and regenerated SF were used for developing nano-fibrous scaffolds through “green electrospinning process” by utilizing less toxic Q3C class-3 solvent [218]. Typically, 15% w/w solutions of PU in formic acid having 0, 10 and 20% SF were prepared. Solutions were placed in 5 mL plastic syringe equipped with 0.8 mm outer diameter blunt needle. Potential difference was applied to solution by Spellman’s CZE1000R high voltage power supply. Feed rate of solutions was controlled by KDS100 infusion pump (KD-Scientific).

Table 7.1. Sample coding, solution concentration, spinning parameters and fibers diameter

Sample code	(15 w/w%) Solutions in FA		Voltage (kV)	Distance (cm)	Feed rate (μ L/min)	Average fiber diameter (nm) n=10
	PU%	SF%				
PU	100	0	17.5	12	6	214 $\pm$ 15
PU/SF-10	90	10	17.5	12	5	209 $\pm$ 30
PU/SF-20	80	20	17.5	12	4	197 $\pm$ 31

Nanofibers were collected on aluminum sheet. Aluminum sheet along-with fibers was soaked in methanol to wash out any residues of formic acid and the composite web produced was peeled off. Scaffolds were first treated with ammonia solution for 1 h to neutralize formic acid residue and then treated with methanol for β -sheet incorporation. After frequently washing with distilled water, scaffolds were dried firstly in hood for overnight and then in vacuum oven at RT until constant weight. Nano-fibrous scaffolds consisting of pure PU and PU/SF were characterized and used. Sample coding, solution concentration and processing parameters for all samples prepared are given in Table 7.1.

7.2.2.3 Characterization techniques

ATR-FTIR spectra of nano-fibrous scaffolds were obtained by using Thermo-scientific iS50 FTIR spectrometer having ATR attachment. Absorption spectra were recorded at 4 cm^{-1} resolutions and 64 scans. Surface topographical analysis of samples was done on Zeiss EVO LS 15 Scanning Electron Microscope (SEM). Prior to analysis, $\sim 10\text{nm}$ gold layer was sputter coated on samples to make surface fairly conductive. Static water contact angle was measured on Dataphysics OCA 35 goniometer. Tensile strength of developed scaffolds was measured on Instron Model 4411 universal testing machine. 25 mm/min crosshead speed was used and analysis was performed at RT. Samples were cut in shape of dog-bone (24 mm in length) with standard cutting die according to ASTM D1708 standard.

7.3 Results and discussion

7.3.1 ATR-FTIR analysis

Qualitative structural analysis of nano-fibrous PU and PU/SF composite scaffolds was obtained by ATR-FTIR spectroscopy. Absorption spectra of SF powder, PU, PU/SF-10 and PU/SF-20 nano-fibrous scaffolds are reproduced in Figure 7.2. In the FTIR spectra of PU, we can see the characteristics very strong ester carbonyl ($\text{C}=\text{O}$) band of PCL at 1722 cm^{-1} .

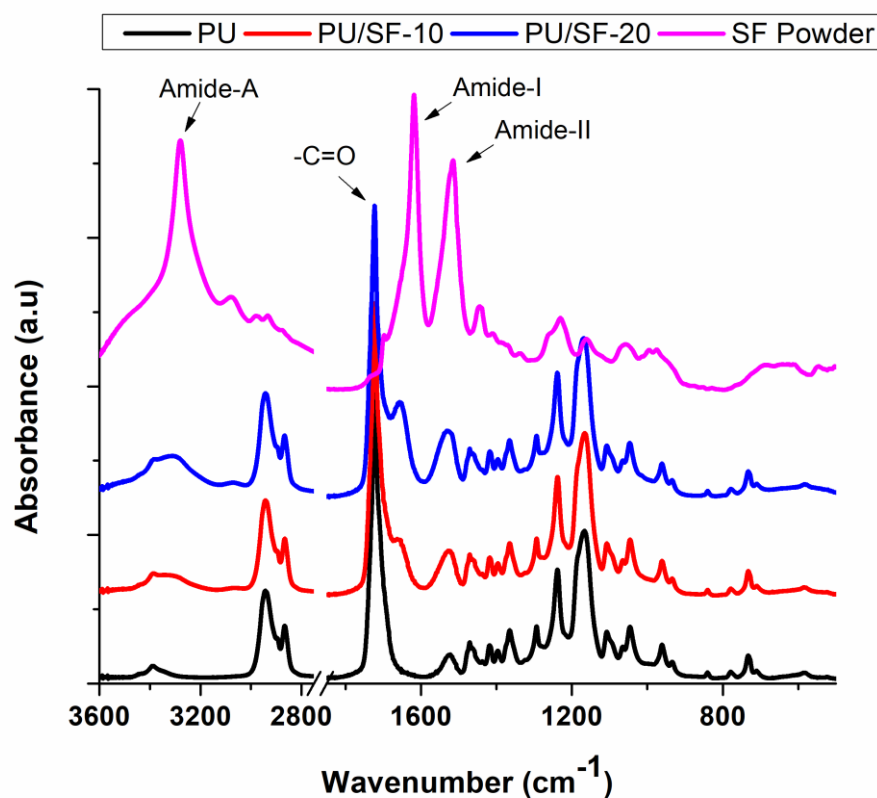


Figure 7.2. ATR-FTIR analysis of SF powder, PU and PU/SF nano-fibrous scaffolds

A small amide band due to urethane group can be seen at 3390 cm^{-1} . In the FTIR spectrum of SF very strong Amide-A (3279 cm^{-1}), Amide-I ($1720\text{--}1580\text{ cm}^{-1}$) and Amide-II ($1580\text{--}1480\text{ cm}^{-1}$) peaks are present. After the incorporation of SF in PU/SF-10 and PU/SF-20 scaffolds, Amide-A band from SF shifts slightly to $\sim 3310\text{ cm}^{-1}$ which clearly shows that there is strong interaction present in PU and SF. More clear evidence of SF incorporation can be seen in Amide-I ($1720\text{--}1580\text{ cm}^{-1}$) and Amide-II regions ($1580\text{--}1480\text{ cm}^{-1}$). In amide-I region, free and H-bonded --C=O stretching band are observed whereas Amide-II region contains --CH stretching and --NH bending vibrations [207]. In Amide-I region, absorption bands corresponding to well-ordered amide ($\text{C=O}\cdots\text{H--N}$) groups responsible for β -sheet formation appear at 1698 and 1618 cm^{-1} in case of SF powder. 1618 cm^{-1} band shifts to 1656 and 1663 cm^{-1} in case of PU/SF-10 and PU/SF-20 respectively. This shows that amide group of SF has interaction with the urethane groups in PU. Band at 1698 cm^{-1} is overlapped with --C=O of PU. In Amide-II region of SF powder, absorption bands appearing at 1505 and 1555 cm^{-1} correspond to β -sheet conformation and band at 1527

cm^{-1} is related to random coil structures. These bands overlap with strong (C-C) stretching band at 1514 cm^{-1} [208]. Due to $-\text{NH}$ bending vibrations present in this region, these bands are shifted to 1528 and 1530 cm^{-1} in case of PU/SF-10 and PU/SF-20 respectively. So, ATR-FTIR analysis show that there is strong interaction present between PU and SF in nano-fibrous composite scaffolds.

7.3.2 Surface analysis

Surface analysis of PU and PU/SF composite scaffolds was performed through SEM and secondary electron (SE) images are provided in Figure 7.3.

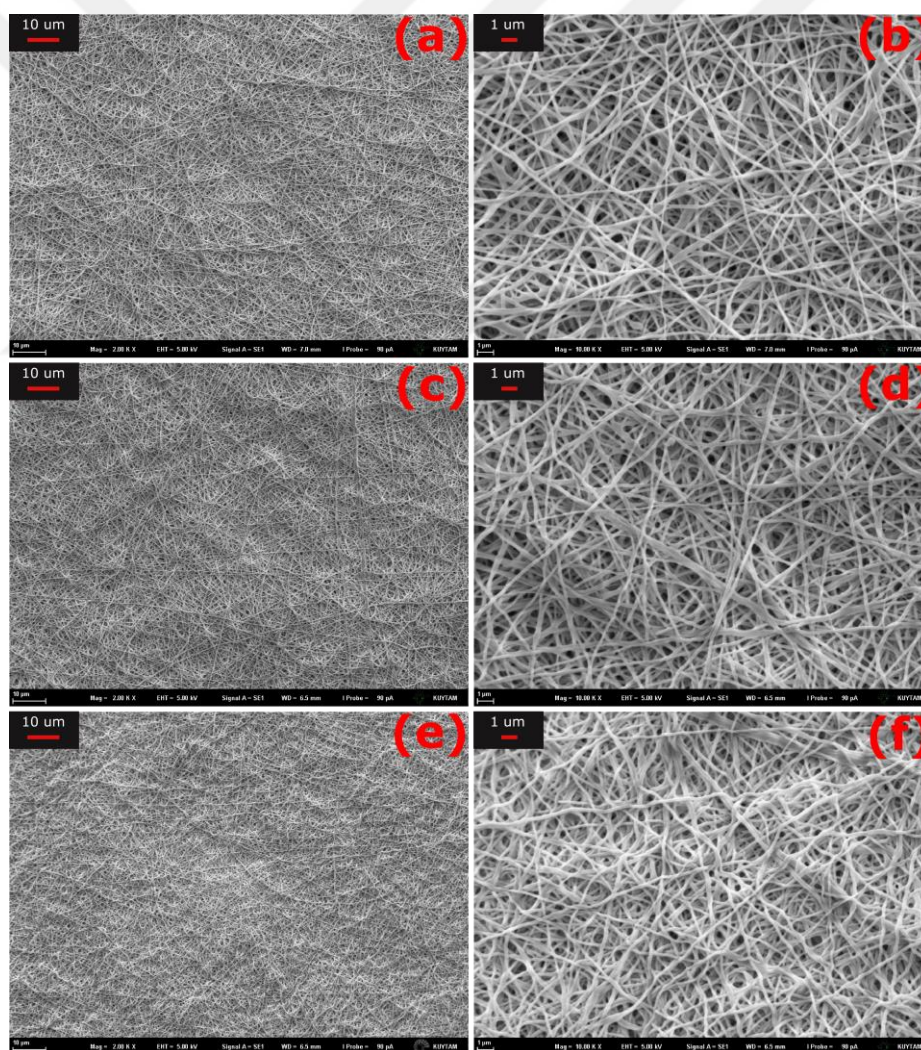


Figure 7.3. SEM analysis of nano-fibrous scaffolds: (a, b) PU, (c, d) PU/SF-10 and (e, f) PU/SF-20

During the electrospinning of all solutions, bead free fibrous networks were obtained. This means that formic acid is a good solvent for electrospinning of these polymeric systems. Diameters of the fibers produced were estimated through ImageJ software and average diameters for all samples are provided in Table 7.1. We can observe that, after SF incorporation, average diameter was slightly lower than pristine PU. Morphological analysis also shows that; fibers tend to attach each other specially in case of PU/SF-20 as can be seen in Figure 7.3-f. This can be due to the hydrogen bonding between urethane and amide groups in PU and SF. This phenomenon also leads to a decrease in the porosity of the scaffold due to the increase in the packing density. Surface wetting properties of all electro-spun scaffolds were also determined through water contact angle measurements. For comparison water contact angles of respective flat films spin coated on glass slides at 1000 rpm for 70 seconds were also determined. Contact angle values and the photographic images of the contact angles are provided in Figure 7.4, for all surfaces. Spin coated PU film surface shows a CA of $74.2 \pm 0.6^\circ$. CA becomes slightly lower after 10% ($70.7 \pm 0.5^\circ$) and 20% ($68 \pm 0.6^\circ$) SF incorporation due to the hydrophilic nature of SF.

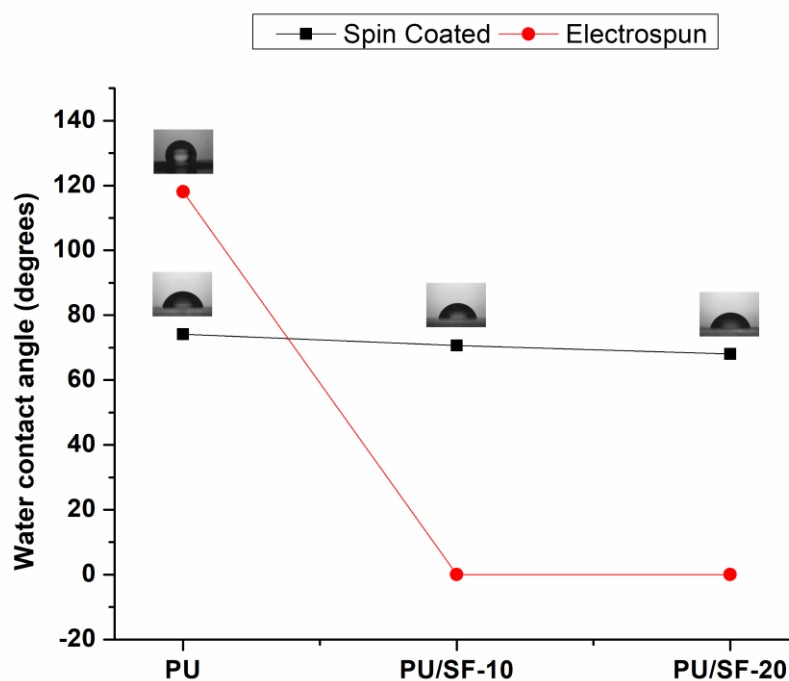


Figure 7.4. Water contact angle measurements on spin coated and electro-spun scaffolds

Quite interesting results were observed when electro-spun scaffolds were analyzed. PU electro-spun scaffolds displayed a CA of $118.1 \pm 0.8^\circ$. This increase in the CA compared with the smooth spin coated film surface is due to the surface roughness of the electro-spun scaffold, which contains air pockets. When SF was incorporated either 10% or 20%, CA becomes almost 0° . This is the same behavior, which was explained in Section 6.3.2. Super-hydrophilic nature obtained of the composite scaffolds can be very beneficial for initial attachment of cells and their growth with time. These surfaces may also restrict the bacterial adhesion and growth as well [224].

7.3.3 Degradation analysis

Scaffolds were analyzed for their hydrolytic degradation behavior. For this purpose, DMEM medium having 10% FBS was used. Samples were put in medium and incubated at 37°C . After 1, 3, 7, 11 and 15 weeks, samples were taken out, washed with warm distilled water and dried in vacuum oven at RT until constant weight. Medium was also changed at each time interval. Remaining weight% against respective time is plotted in Figure 7.5.

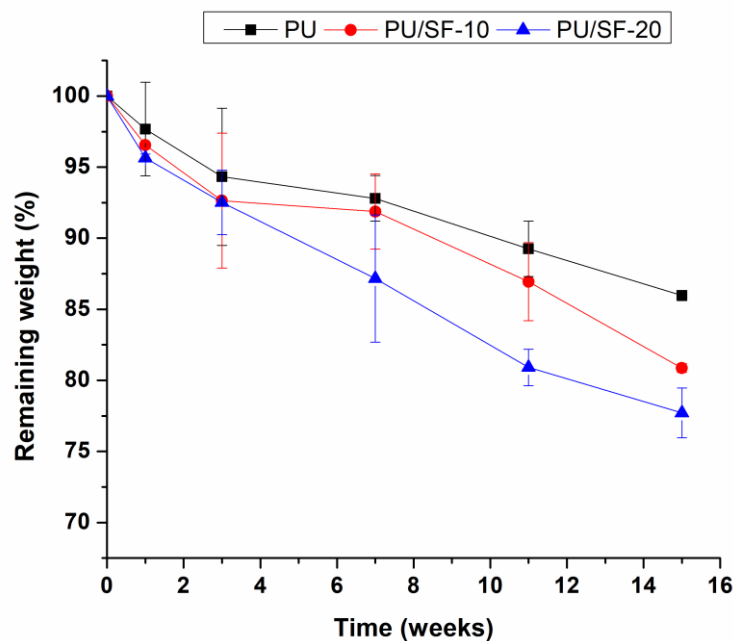


Figure 7.5. Remaining weight of nano-fibrous scaffolds against time

There was a gradual decrease in weight of PU scaffold observed. The decrease in weight after 15 weeks of incubation was ~14%. This degradation is possibly due to highly polar urethane groups present in PU. After incorporation of SF, a higher rate of decrease in weight was observed. In case of PU/SF-20, loss in weight was ~22% after 15 weeks. We can say that SF is selectively more prone to degradation in FBS containing DMEM medium than pristine PU and this controlled degradation can be tuned and optimized with SF concentration for tissue regeneration applications. However, enzymatic degradation causes more loss in weight% [225].

7.3.4 Mechanical properties of scaffolds

Mechanical properties of scaffolds were determined by using universal testing machine. Ultimate tensile strength (UTS), elongation at break and elastic modulus (EM) values of PU and PU/SF composite scaffolds are compared with the values of natural human skin obtained from literature [221]. Results are presented in Figure 7.6.

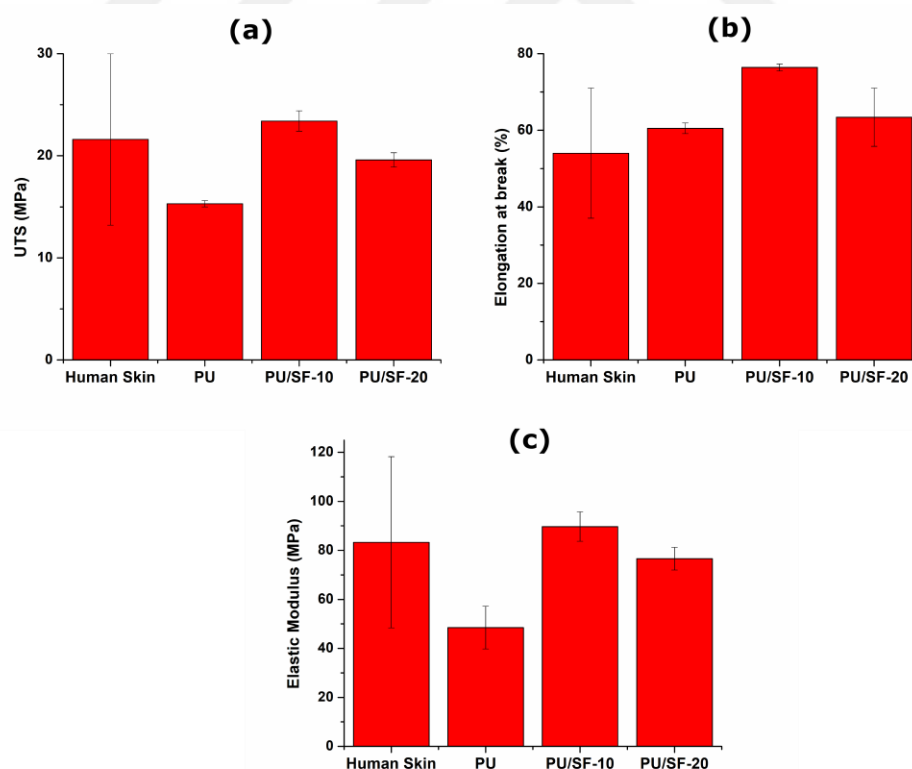


Figure 7.6. Mechanical properties comparison of PU, PU/SF and human skin: a) ultimate tensile strength, b) elongation at break and c) elastic modulus

Ultimate tensile strength of 15.3 ± 0.3 MPa, elongation at break of $60.5 \pm 1.4\%$ and elastic modulus of 48.5 ± 8.7 MPa was observed for PU scaffold. After SF addition in PU matrix either as 10 or 20 wt%, UTS, elongation and EM values were enhanced. This can be due to the strong interaction and H-bonding present between urethane and amide groups present in PU and SF. Most importantly, all the mechanical properties of PU/SF composite scaffolds are almost similar to native human skin. Hence, these PU/SF composite scaffolds can be better choice for skin substitutes.

7.3.5 *In-vitro cells viability analysis*

For cell viability analysis, NIH-3T3-mouse embryo fibroblast cells were used. Firstly, cells were grown in DMEM media having L-glutamine, 10% FBS and 5% penicillin-streptomycin. Cells were detached from plate by using 1 mL of 0.5% Trypsin-EDTA solution, incubated plate at 37 °C for 2 minutes, added 4 mL of low glucose DMEM (containing 10% FBS and 1% P/S) and gently mixed. For cells counting, 10 μ L of this mixture was pipetted out and poured in hemocytometer. While the cells were counted under inverted microscope at 10X magnification, rest of cells suspension was taken in a falcon tube and centrifuged for 5 minutes at 250 g. Supernatant was aspirated and required amount of high glucose DMEM medium having 10% FBS and 5% P/S was added to make cells as 6000/well for 96 well plate. Scaffolds were cut in 6 mm diameter and UV sterilized from both sides for half an hour each. 100 μ L of cell suspension was added carefully on scaffolds and control (n=5). 96 well plates for both 1st and 4th day analysis were incubated in culture incubator at 37 °C and having 5% CO₂. The cytotoxicity of scaffolds was determined by using MTT assay and results are provided in Figure 7.7. Analysis was performed on Day-1 and Day-4 (cells seeding on samples is named as Day-0). MTT stock solution in complete medium was prepared as 1 mg/mL. On Day-1 and Day-4, firstly, medium was aspirated from 96 well plate and 100 μ L of solution containing MTT (0.5 mg/mL) and DMEM at ratio of 1:4 was added in each well. Well plates were again incubated at 37 °C and 5% CO₂ atmosphere for 3.5 h. After that, supernatant was removed, and dark blue crystals of formazan were dissolved in 200 μ L mixture of ethanol/DMSO (1:1 by volume). The appropriate amount of this solution was transferred to 96 well plate and optical densities were read at 600 and 690 nm in Biotek Synergy plate reader.

Absorbance at 690 nm was taken as background. MTT assay results against time are given in Figure 7.7.

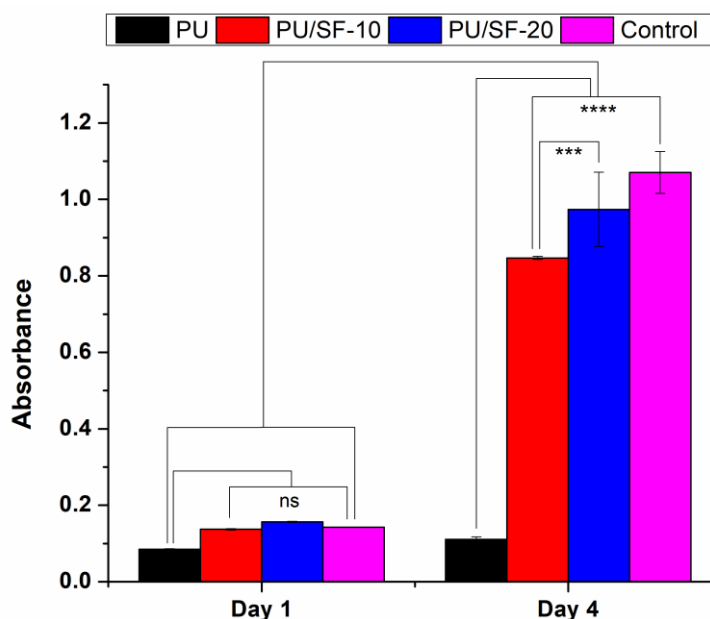


Figure 7.7. MTT analysis of scaffolds after 1st and 4th day of osteosarcoma cell seeding

Two-way ANOVA analysis followed by Tukey's multiple comparison test was performed to analyze the results. Results show that increase in number of cells on composite scaffolds (Day-4) is quite substantial and significant (**** $p < 0.0001$) than Day-1 and non-significant on PU. Overall, we can conclude that, scaffolds showed no toxicity to cells and incorporation of SF resulted in much enhanced proliferation of fibroblast cells. Increase in number of cells over the time was also observed through SEM analysis. On Day-1 and Day-4, cells on scaffolds were fixed through 2.5 v/v% solution of glutaraldehyde (GA) in PBS. After 3 h at RT and 24 h at 4 °C incubation, GA was removed and samples were dehydrated with increasing gradient concentration of ethanol (30, 50, 70, 90 and 100%) for 3 h each. Then scaffolds were dried in hood and then in vacuum oven at RT. Scaffolds were pre-coated with ~10 nm gold layer through sputter coating machine and then SE images were obtained and provided in Figure 7.8. Figures 7.8(a, b, c) represent PU, 7.8(d, e, f) shows PU/SF-10 and 7.8(g, h, i) denote PU/SF-20 scaffolds. Similarly, Figures 7.8(a, d, g) and 7.8(b, c, e, f, h, i) correspond to Day-1 and Day-4 respectively.

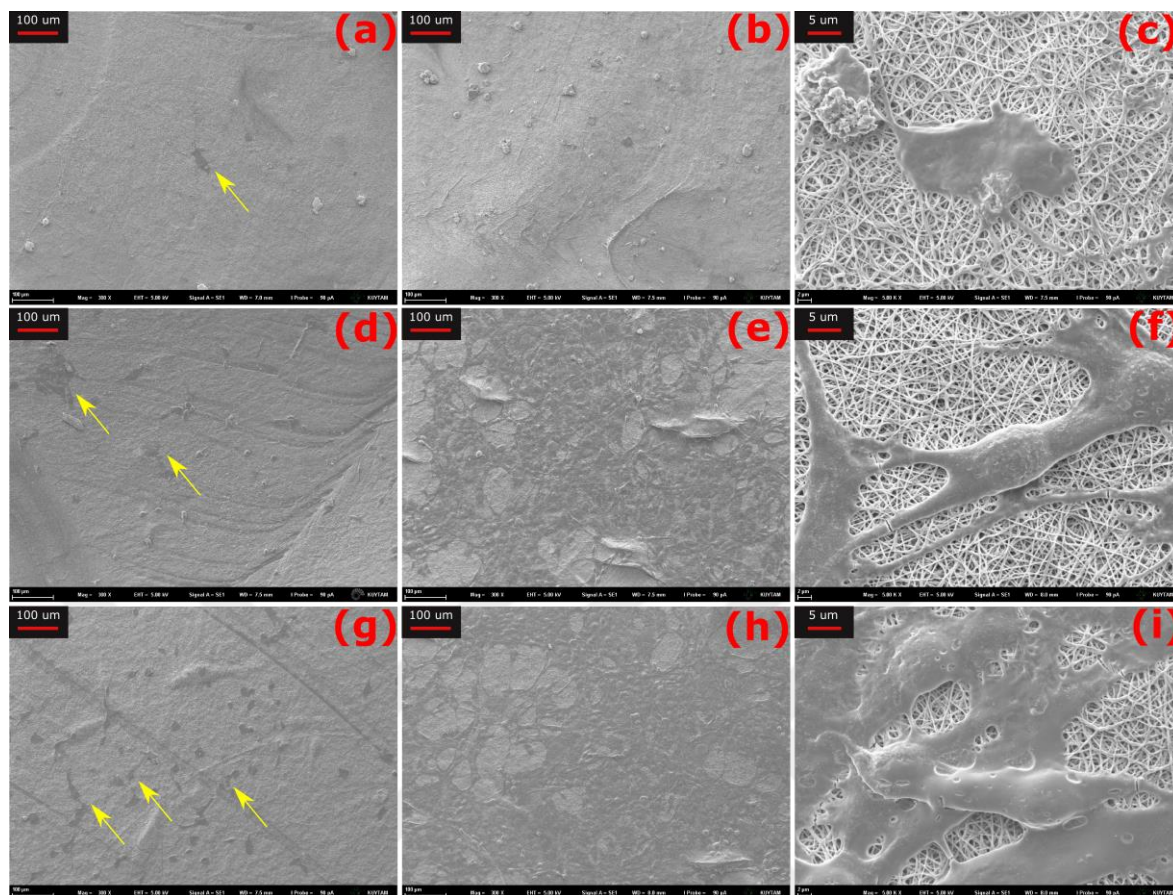


Figure 7.8. SEM analysis of scaffolds after cells seeding: PU (a, b, c), PU/SF-10 (d, e, f) and PU/SF-20 (g, h, i).

On day-1, pristine PU scaffold I having less no of cells as compared to PU/SF as indicated by yellow arrows. On day-4, small increase in number of cells was observed on PU scaffold while, almost the whole surfaces of PU/SF scaffolds are covered by cells. These results are also in accordance with the MTT analysis. Morphology of cells on PU, PU/SF-10 and PU/SF-20 can be seen in Figures 7.8(c, f and i) respectively. Cells are more spread and attached on composite scaffolds due to SF presence in the system.

7.4 Conclusions

In this study, silk (*Bombyx mori*) cocoons were degummed through novel process by treating cocoons in 0.3 w/w% ammonia solution. These degummed fibers were dissolved in formic acid/phosphoric acid (7:3) mixture by volume. Fiber solution was coagulated in methanol and the precipitate was filtered, washed and dried to obtain pure SF. Novel

polyurethane comprised of PCL2k and HDI (1:1 by mol.) was synthesized in THF at reflux. After purifying, PU was used to make nano-fibrous scaffolds with and without SF through electrospinning. Formic acid was found to be a good solvent for preparing well defined nano-fibrous scaffolds as shown by SEM. ATR-FTIR analysis confirmed successful incorporation of SF in composite scaffolds. These biodegradable scaffolds were found to be non-toxic to cells through *in-vitro* seeding of NIH-3T3-mouse embryo fibroblast cells. PU/SF composite scaffolds resulted in enhanced proliferation of cells as determined by MTT assay. Proliferation of cells was almost 8 folds in composite scaffold than PU. Morphological analysis of the cells shows enhanced spreading and attachment of cells on composite scaffolds as compared to pristine PU scaffolds. We believe that enhanced biomechanical properties of PU/SF composite scaffolds having good mechanical, elastic properties and bioactivity offer potential applications in skin tissue engineering applications and can be used as skin substitutes.

CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

In this work, biodegradable scaffolds have been developed for bone and skin tissue engineering applications through green processes. Biomaterials such as hydroxyapatite (HA), chitosan (CS) and poly(lactic acid) (PLA) were studied for bone tissue engineering applications. HA was synthesized in our laboratories. Different production routes were employed during HA synthesis and synthetic process was analyzed through various techniques. Different solvent media like water, THF and DMF were used to see the impact of dielectric constant (DC) of reaction mixture on HA synthesis. It was concluded that, THF based system having lowest DC produced pure HA while other routes also yielded β -CPF along-with HA as well. HA was used to develop nano-composites with CS by simple solution casting method. Formic acid was used as a solvent due to stable HA dispersions and being less toxic. Due to better dispersion of HA particles in CS solution, developed composites resulted in intercalated structures of CS due to the presence of individual HA particles in between CS chains. Moreover, 3D scaffolds of PLA through fused deposition modeling were also developed by using low cost desktop printer. PLA surface was modified with CS/HA dispersions in formic acid through dipping and quenching method. Formic acid caused PLA chains to swell so CS/HA was successfully immobilized on PLA surfaces and partial filling of macro pores as well. Scaffolds were characterized through different techniques confirming the incorporation and interaction among PLA, CS and HA. Toxicity of these developed scaffolds was checked through in-vitro seeding of U2-OS human osteosarcoma cells. All scaffolds were found to be non-toxic and MTT assay proved enhanced proliferation of cells on modified PLA scaffolds. Degradation studies showed faster degradation of CS followed by slower degradation of PLA in DMEM media at physiological conditions. Slower degradation of PLA can be

useful since it will provide longer period for bones to regenerate. Hence, these scaffolds can be used for bone defect repair and regeneration.

PCL, PU and SF were employed and characterized for potential skin substitutes. PCL was synthesized in our laboratories through ROP of ϵ -caprolactone in the presence of T-9 catalyst by using 1,2-bis(3-aminopropoxy)ethane as initiator. Reaction was carried out in xylene at $130\pm 5^\circ\text{C}$. Synthesized PCL was purified to remove any unreacted moieties and catalyst by coagulation in methanol prior to use. Novel PU was synthesized through step growth polymerization of PCL2k and HDI in 1:1 mole ratio in catalytic presence of T-12. Reaction was carried out in THF at reflux. Purification was performed by coagulation of polymer solution in methanol/water (1:1) mixture. Obtained polymer was used further to develop scaffolds. Regenerated SF was obtained through various degumming processes. Firstly, cocoons were degummed through 0.3 w/w% solutions of Na_2CO_3 , NaHCO_3 and NH_4OH solutions at boiling for 45, 60 and 90 minutes. Degummed fibers were dissolved in formic/phosphoric acid (7:3), coagulated in methanol and SF was obtained after washing and drying. Obtained SF through various processes was characterized through different techniques. It was concluded that, degumming reagent and processing has significant effect on conformational composition of SF. Therefore, SF obtained through ammonia solution based degumming was used in further processes to develop scaffolds. Nano-fibrous scaffolds of PCL and PU having different weight% of SF were obtained through “green electrospinning process” at appropriate conditions. Formic acid, a Q3C class 3 solvent as defined by FDA was used in developing scaffolds. SF was added in PCL solution in formic acid as 20 and 40 weight% while in PU as 10 and 20 weight%. Successful incorporation of SF was confirmed through various analyses. PCL and PU electro-spun surfaces having water contact angles of $129.1\pm 1.3^\circ$ and $118.1\pm 0.8^\circ$ respectively became super-hydrophilic after SF incorporation. Human BJ fibroblast cells were seeded on PCL and PCL/SF composite scaffolds. ATP analysis revealed that proliferation was enhanced after SF incorporation. Almost 97% more proliferation was observed on 4th Day analysis on PCL/SF-40 surface as compared to pristine PCL. Similarly, NIH/3T3-Mouse embryo fibroblast cells were seeded on PU and PU/SF composites. Much enhanced proliferation was observed through MTT analysis on PU/SF composite scaffolds. On 4th Day analysis, proliferation was $\sim 775\%$ than pristine PU scaffold. This was also confirmed through SEM analysis of cell containing scaffolds.

Almost the whole surface was covered with cells on 4th Day in case of composite scaffolds. Incorporation of SF also accelerated degradation of scaffolds. Mechanical analysis show that these composite scaffolds have good enough mechanical properties comparable to native human skin. So conclusively, we can say that our developed composite scaffolds can be used as skin substitutes for skin tissue engineering and regenerative medicine applications.

In this thesis study, a variety of biodegradable scaffolds were developed through the use of novel materials and techniques. *In-vitro* cell seeding analyses clearly demonstrated that these scaffolds are non-toxic and bioactive, resulting in enhanced cell growth on these surfaces. As future work, these scaffolds can be used for *in-vivo* analysis and post implantation process, immunological impact can be assessed and determined on vital organs.

BIBLIOGRAPHY

1. Langer, R. and D.A. Tirrell, *Designing materials for biology and medicine*. Nature, 2004. **428**(6982): p. 487.
2. Bhullar, S.K., et al., *Design and fabrication of auxetic PCL nanofiber membranes for biomedical applications*. Materials Science & Engineering C-Materials for Biological Applications, 2017. **81**: p. 334-340.
3. Jeon, H. and G. Kim, *Preparation and characterization of an electrospun polycaprolactone (PCL) fibrous mat and multi-layered PCL scaffolds having a nanosized pattern-surface for tissue regeneration*. Journal of Materials Chemistry B, 2014. **2**(2): p. 171-180.
4. Reid, L., et al., *Regulation of growth and differentiation of epithelial cells by hormones, growth factors, and substrates of extracellular matrix*. Annals of the New York Academy of Sciences, 1981. **372**(1): p. 354-370.
5. Furth, M.E., A. Atala, and M.E. Van Dyke, *Smart biomaterials design for tissue engineering and regenerative medicine*. Biomaterials, 2007. **28**(34): p. 5068-5073.
6. Hassani, A., et al., *Measurement of volume changes of sinus floor augmentation covered with buccal fat pad: a case series study*. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics, 2009. **107**(3): p. 369-374.
7. Hassani, A., A. Khojasteh, and A.N. Shamsabad, *The anterior palate as a donor site in maxillofacial bone grafting: a quantitative anatomic study*. Journal of oral and maxillofacial surgery, 2005. **63**(8): p. 1196-1200.
8. Phinney, D.G. and D.J. Prockop, *Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair—current views*. Stem cells, 2007. **25**(11): p. 2896-2902.
9. Behnia, H., et al., *Secondary repair of alveolar clefts using human mesenchymal stem cells*. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics, 2009. **108**(2): p. e1-e6.
10. Behnia, H., et al., *Repair of alveolar cleft defect with mesenchymal stem cells and platelet derived growth factors: a preliminary report*. Journal of Cranio-Maxillo-Facial Surgery, 2012. **40**(1): p. 2-7.
11. Takahashi, Y., M. Yamamoto, and Y. Tabata, *Osteogenic differentiation of mesenchymal stem cells in biodegradable sponges composed of gelatin and β -tricalcium phosphate*. Biomaterials, 2005. **26**(17): p. 3587-3596.
12. Zhong, S., Y. Zhang, and C. Lim, *Tissue scaffolds for skin wound healing and dermal reconstruction*. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 2010. **2**(5): p. 510-525.
13. Gurtner, G.C., et al., *Wound repair and regeneration*. Nature, 2008. **453**(7193): p. 314.
14. Pastar, I., et al., *Epithelialization in wound healing: a comprehensive review*. Advances in wound care, 2014. **3**(7): p. 445-464.

15. Shakespeare, P., *Burn wound healing and skin substitutes*. Burns, 2001. **27**(5): p. 517-522.
16. Lee, C.H., A. Singla, and Y. Lee, *Biomedical applications of collagen*. International journal of pharmaceutics, 2001. **221**(1-2): p. 1-22.
17. Jung, J.-A., et al., *Evaluation of the efficacy of highly hydrophilic polyurethane foam dressing in treating a diabetic foot ulcer*. Advances in skin & wound care, 2016. **29**(12): p. 546-555.
18. Augustine, R., N. Kalarikkal, and S. Thomas, *Electrospun PCL membranes incorporated with biosynthesized silver nanoparticles as antibacterial wound dressings*. Applied Nanoscience, 2016. **6**(3): p. 337-344.
19. Izadifar, Z., X. Chen, and W. Kulyk, *Strategic design and fabrication of engineered scaffolds for articular cartilage repair*. Journal of functional biomaterials, 2012. **3**(4): p. 799-838.
20. Huétink, K., et al., *Identification of factors associated with the development of knee osteoarthritis in a young to middle-aged cohort of patients with knee complaints*. Clinical rheumatology, 2015. **34**(10): p. 1769-1779.
21. Zhou, Y., J. Chyu, and M. Zumwalt, *Recent Progress of Fabrication of Cell Scaffold by Electrospinning Technique for Articular Cartilage Tissue Engineering*. International journal of biomaterials, 2018. **2018**.
22. Lawson, J.H., et al., *Bioengineered human acellular vessels for dialysis access in patients with end-stage renal disease: two phase 2 single-arm trials*. The Lancet, 2016. **387**(10032): p. 2026-2034.
23. Hibino, N., et al., *Late-term results of tissue-engineered vascular grafts in humans*. The Journal of thoracic and cardiovascular surgery, 2010. **139**(2): p. 431-436. e2.
24. Dahl, S.L., et al., *Readily available tissue-engineered vascular grafts*. Science translational medicine, 2011. **3**(68): p. 68ra9-68ra9.
25. Syedain, Z.H., et al., *Implantation of completely biological engineered grafts following decellularization into the sheep femoral artery*. Tissue Engineering Part A, 2014. **20**(11-12): p. 1726-1734.
26. Black, J., *Biological performance of materials: fundamentals of biocompatibility*. 2005: Crc Press.
27. Iqbal, N., et al., *Recent concepts in biodegradable polymers for tissue engineering paradigms: a critical review*. International Materials Reviews, 2018: p. 1-36.
28. Novotna, K., et al., *Poly(lactide) nanofibers with hydroxyapatite as growth substrates for osteoblast-like cells*. Journal of Biomedical Materials Research Part A, 2014. **102**(11): p. 3918-3930.
29. Rogina, A., et al., *Macroporous poly(lactic acid) construct supporting the osteoinductive porous chitosan-based hydrogel for bone tissue engineering*. Polymer, 2016. **98**: p. 172-181.
30. LogithKumar, R., et al., *A review of chitosan and its derivatives in bone tissue engineering*. Carbohydrate Polymers, 2016. **151**: p. 172-188.
31. Boskey, A.L., *Bone composition: relationship to bone fragility and antiosteoporotic drug effects (vol 2, 447, 2013)*. Bonekey Reports, 2015. **4**.

32. Wopenka, B. and J.D. Pasteris, *A mineralogical perspective on the apatite in bone*. Materials Science & Engineering C-Biomimetic and Supramolecular Systems, 2005. **25**(2): p. 131-143.
33. Link, T., et al., *An approach to a biomimetic bone scaffold: increased expression of BMP-2 and of osteoprotegerin in SaOS-2 cells grown onto silica-biologized 3D printed scaffolds*. Rsc Advances, 2013. **3**(28): p. 11140-11147.
34. Sambudi, N.S., S. Cho, and K. Cho, *Porous hollow hydroxyapatite microspheres synthesized by spray pyrolysis using a microalga template: preparation, drug delivery, and bioactivity*. Rsc Advances, 2016. **6**(49): p. 43041-43048.
35. Cellet, T.S.P., et al., *Hydroxyapatite nanowhiskers embedded in chondroitin sulfate microspheres as colon targeted drug delivery systems*. Journal of Materials Chemistry B, 2015. **3**(33): p. 6837-6846.
36. Zeitz, C., et al., *Synthesis of Hydroxyapatite Substrates: Bridging the Gap between Model Surfaces and Enamel*. Acs Applied Materials & Interfaces, 2016. **8**(39): p. 25848-25855.
37. Raucci, M.G., et al., *Properties of carbon nanotube-dispersed Sr-hydroxyapatite injectable material for bone defects*. Regenerative Biomaterials, 2016. **3**(1): p. 13-23.
38. Huang, Y.X., et al., *An injectable nano-hydroxyapatite (n-HA)/glycol chitosan (G-CS)/hyaluronic acid (HyA) composite hydrogel for bone tissue engineering*. Rsc Advances, 2016. **6**(40): p. 33529-33536.
39. Mistry, S., et al., *Clinical Outcome of Hydroxyapatite Coated, Bioactive Glass Coated, and Machined Ti6Al4V Threaded Dental Implant in Human Jaws: A Short-Term Comparative Study*. Implant Dentistry, 2016. **25**(2): p. 252-260.
40. Silva, D.F., et al., *Characterization of mesoporous calcium phosphates from calcareous marine sediments containing Si, Sr and Zn for bone tissue engineering*. Journal of Materials Chemistry B, 2016. **4**(42): p. 6842-6855.
41. Haider, A., et al., *Recent advances in the synthesis, functionalization and biomedical applications of hydroxyapatite: a review*. Rsc Advances, 2017. **7**(13): p. 7442-7458.
42. Nair, A.K., A. Gautieri, and M.J. Buehler, *Role of Intrafibrillar Collagen Mineralization in Defining the Compressive Properties of Nascent Bone*. Biomacromolecules, 2014. **15**(7): p. 2494-2500.
43. Nair, A.K., et al., *Molecular mechanics of mineralized collagen fibrils in bone*. Nature Communications, 2013. **4**.
44. Gentile, P., et al., *Process Optimisation to Control the Physico-Chemical Characteristics of Biomimetic Nanoscale Hydroxyapatites Prepared Using Wet Chemical Precipitation*. Materials, 2015. **8**(5): p. 2297-2310.
45. Houmard, M., et al., *Sol-gel method to fabricate CaP scaffolds by robocasting for tissue engineering*. Journal of Materials Science-Materials in Medicine, 2012. **23**(4): p. 921-930.
46. Li, X.N., et al., *Synthesis of Hydroxyapatite from Cuttlefish Bone via Hydrothermal Solid-State Conversion*. Journal of Biomaterials and Tissue Engineering, 2016. **6**(10): p. 775-779.
47. Li, H., et al., *Synthesis and cytotoxicity of carbon nanotube/hydroxyapatite in situ composite powders prepared by chemical vapour deposition*. Materials Research Innovations, 2014. **18**: p. 338-343.

48. Cunniffe, G.M., et al., *The synthesis and characterization of nanophase hydroxyapatite using a novel dispersant-aided precipitation method*. Journal of Biomedical Materials Research Part A, 2010. **95A**(4): p. 1142-1149.
49. Owens, G.J., et al., *Sol-gel based materials for biomedical applications*. Progress in Materials Science, 2016. **77**: p. 1-79.
50. Pang, Y.X. and X. Bao, *Influence of temperature, ripening time and calcination on the morphology and crystallinity of hydroxyapatite nanoparticles*. Journal of the European Ceramic Society, 2003. **23**(10): p. 1697-1704.
51. Abdel-Fattah, W.I., F.M. Reicha, and T.A. Elkhooly, *Characterization of nano-biphasic calcium phosphates synthesized under microwave curing*. Journal of Nano Research, 2008. **3**: p. 67-87.
52. David S. H. Lee, Y.P., Steve Chang, *Effect of Thermal Treatment of the Hydroxyapatite Powders on the Micropore and Microstructure of Porous Biphasic Calcium Phosphate Composite Granules*. Journal of Biomaterials and Nanobiotechnology, 2013. **4**: p. 114-118.
53. Sanosh, K.P., et al., *Preparation and characterization of nano-hydroxyapatite powder using sol-gel technique*. Bulletin of Materials Science, 2009. **32**(5): p. 465-470.
54. Cho, J.S., et al., *Effects of solvent on the properties of nano-sized hydroxyapatite powders directly prepared by high temperature flame spray pyrolysis*. Journal of Ceramic Processing Research, 2009. **10**(5): p. 628-632.
55. Deng, Y., et al., *Nano-hydroxyapatite reinforced polyphenylene sulfide biocomposite with superior cytocompatibility and in vivo osteogenesis as a novel orthopedic implant*. Rsc Advances, 2017. **7**(1): p. 559-573.
56. Rogina, A., et al., *Macroporous poly(lactic acid) construct supporting the osteoinductive porous chitosan-based hydrogel for bone tissue engineering*. Polymer, 2016. **98**: p. 172-181.
57. Levengood, S.K.L. and M.Q. Zhang, *Chitosan-based scaffolds for bone tissue engineering*. Journal of Materials Chemistry B, 2014. **2**(21): p. 3161-3184.
58. Muthukumar, T., et al., *Collagen/chitosan porous bone tissue engineering composite scaffold incorporated with Ginseng compound K*. Carbohydrate Polymers, 2016. **152**: p. 566-574.
59. Kim, H.W., J.H. Song, and H.E. Kim, *Nanoriber generation of gelatin-hydroxyapatite biomimetics for guided tissue regeneration*. Advanced Functional Materials, 2005. **15**(12): p. 1988-1994.
60. Hassan, M.I., T. Sun, and N. Sultana, *Fabrication of Nanohydroxyapatite/Poly(caprolactone) Composite Microfibers Using Electrospinning Technique for Tissue Engineering Applications*. Journal of Nanomaterials, 2014.
61. Wang, Z.L., et al., *A comparative study on the in vivo degradation of poly(L-lactide) based composite implants for bone fracture fixation*. Scientific Reports, 2016. **6**.
62. Prajatelista, E., et al., *Chitosan and hydroxyapatite composite cross-linked by dopamine has improved anisotropic hydroxyapatite growth and wet mechanical properties*. Engineering in Life Sciences, 2015. **15**(2): p. 254-261.
63. Zhang, X.Q., et al., *The effect of the prefrozen process on properties of a chitosan/hydroxyapatite/poly(methyl methacrylate) composite prepared by freeze*

- drying method used for bone tissue engineering. *Rsc Advances*, 2015. **5**(97): p. 79679-79686.
64. Kang, M.H., et al., *Poly(ether imide)-silica hybrid coatings for tunable corrosion behavior and improved biocompatibility of magnesium implants*. *Biomedical Materials*, 2016. **11**(3).
 65. Suh, J.-K.F. and H.W. Matthew, *Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review*. *Biomaterials*, 2000. **21**(24): p. 2589-2598.
 66. Jin, H.J. and D.L. Kaplan, *Mechanism of silk processing in insects and spiders*. *Nature*, 2003. **424**(6952): p. 1057-1061.
 67. Wang, H.Y. and Y.Q. Zhang, *Processing and characterisation of a novel electropolymerized silk fibroin hydrogel membrane*. *Scientific Reports*, 2014. **4**.
 68. Wang, J.N., et al., *Cytocompatibility of a silk fibroin tubular scaffold*. *Materials Science & Engineering C-Materials for Biological Applications*, 2014. **34**: p. 429-436.
 69. Vepari, C. and D.L. Kaplan, *Silk as a biomaterial*. *Progress in Polymer Science*, 2007. **32**(8-9): p. 991-1007.
 70. Kim, H.H., et al., *Effect of silk fibroin molecular weight on physical property of silk hydrogel*. *Polymer*, 2016. **90**: p. 26-33.
 71. Zhou, L., et al., *Preparation and characterization of transparent silk fibroin/cellulose blend films*. *Polymer*, 2013. **54**(18): p. 5035-5042.
 72. Teimouri, A., et al., *Preparation, characterization, degradation and biocompatibility of different silk fibroin based composite scaffolds prepared by freeze-drying method for tissue engineering application*. *Polymer Degradation and Stability*, 2015. **121**: p. 18-29.
 73. Koh, L.D., et al., *Structures, mechanical properties and applications of silk fibroin materials*. *Progress in Polymer Science*, 2015. **46**: p. 86-110.
 74. Zhou, C.Z., et al., *Fine organization of Bombyx mori fibroin heavy chain gene*. *Nucleic Acids Research*, 2000. **28**(12): p. 2413-2419.
 75. Inoue, S., et al., *Silk fibroin of Bombyx mori is secreted, assembling a high molecular mass elementary unit consisting of H-chain, L-chain, and P25, with a 6 : 6 : 1 molar ratio*. *Journal of Biological Chemistry*, 2000. **275**(51): p. 40517-40528.
 76. Zhou, C.Z., et al., *Silk fibroin: Structural implications of a remarkable amino acid sequence*. *Proteins-Structure Function and Genetics*, 2001. **44**(2): p. 119-122.
 77. Wray, L.S., et al., *Effect of processing on silk-based biomaterials: Reproducibility and biocompatibility*. *Journal of Biomedical Materials Research Part B-Applied Biomaterials*, 2011. **99B**(1): p. 89-101.
 78. Valluzzi, R., et al., *Orientation of silk III at the air-water interface*. *International Journal of Biological Macromolecules*, 1999. **24**(2-3): p. 237-242.
 79. Sashina, E.S., et al., *Structure and solubility of natural silk fibroin*. *Russian Journal of Applied Chemistry*, 2006. **79**(6): p. 869-876.
 80. Allardyce, B.J., et al., *The impact of degumming conditions on the properties of silk films for biomedical applications*. *Textile Research Journal*, 2016. **86**(3): p. 275-287.

81. Ho, M.P., H. Wang, and K.T. Lau, *Effect of degumming time on silkworm silk fibre for biodegradable polymer composites*. Applied Surface Science, 2012. **258**(8): p. 3948-3955.
82. Perez-Rigueiro, J., et al., *Effect of degumming on the tensile properties of silkworm (Bombyx mori) silk fiber*. Journal of Applied Polymer Science, 2002. **84**(7): p. 1431-1437.
83. Cao, T.T., Y.J. Wang, and Y.Q. Zhang, *Effect of Strongly Alkaline Electrolyzed Water on Silk Degumming and the Physical Properties of the Fibroin Fiber*. Plos One, 2013. **8**(6).
84. Teimouri, A., et al., *Nano-composite of silk fibroin-chitosan/Nano ZrO₂ for tissue engineering applications: Fabrication and morphology*. International Journal of Biological Macromolecules, 2015. **76**: p. 292-302.
85. Mahadevaiah, et al., *Effect of Microwave Irradiation on the Microstructural Properties of Bivoltine Silk Fibroin Films*, in *International Conference on Materials for Advanced Technologies*, G.K. Dalapati, et al., Editors. 2016. p. 53-58.
86. Zhu, J.X., et al., *Electrospinning and rheology of regenerated Bombyx mori silk fibroin aqueous solutions: The effects of pH and concentration*. Polymer, 2008. **49**(12): p. 2880-2885.
87. Delezuka, J.A.M., et al., *Silk fibroin organization induced by chitosan in layer-by-layer films: Application as a matrix in a biosensor*. Carbohydrate Polymers, 2017. **155**: p. 146-151.
88. Lai, G.J., et al., *Composite chitosan/silk fibroin nanofibers for modulation of osteogenic differentiation and proliferation of human mesenchymal stem cells*. Carbohydrate Polymers, 2014. **111**: p. 288-297.
89. Uchida, R., et al., *Effect of plasma-irradiated silk fibroin in bone regeneration*. Journal of Bioscience and Bioengineering, 2014. **118**(3): p. 333-340.
90. Chen, C., et al., *Preparation of non-woven mats from all-aqueous silk fibroin solution with electrospinning method*. Polymer, 2006. **47**(18): p. 6322-6327.
91. Mathur, A.B., et al., *The dissolution and characterization of Bombyx mori silk fibroin in calcium nitrate methanol solution and the regeneration of films*. Biopolymers, 1997. **42**(1): p. 61-74.
92. Ha, S.W., Y.H. Park, and S.M. Hudson, *Dissolution of Bombyx mori silk fibroin in the calcium nitrate tetrahydrate-methanol system and aspects of wet spinning of fibroin solution*. Biomacromolecules, 2003. **4**(3): p. 488-496.
93. Kim, H., et al., *Mechanically-reinforced electrospun composite silk fibroin nanofibers containing hydroxyapatite nanoparticles*. Materials Science & Engineering C-Materials for Biological Applications, 2014. **40**: p. 324-335.
94. Min, B.M., et al., *Electrospinning of silk fibroin nanofibers and its effect on the adhesion and spreading of normal human keratinocytes and fibroblasts in vitro*. Biomaterials, 2004. **25**(7-8): p. 1289-1297.
95. Ohgo, K., et al., *Preparation of non-woven nanofibers of Bombyx mori silk, Samia cynthia ricini silk and recombinant hybrid silk with electrospinning method*. Polymer, 2003. **44**(3): p. 841-846.
96. Kim, D.K., B.R. Sim, and G. Khang, *Nature-Derived Aloe Vera Gel Blended Silk Fibroin Film Scaffolds for Cornea Endothelial Cell Regeneration and*

- Transplantation*. *Acs Applied Materials & Interfaces*, 2016. **8**(24): p. 15160-15168.
97. Lv, Q., et al., *Preparation of insoluble fibroin films without methanol treatment*. *Journal of Applied Polymer Science*, 2005. **96**(6): p. 2168-2173.
 98. Lv, Q.A., et al., *Three-dimensional fibroin/collagen scaffolds derived from aqueous solution and the use for HepG2 culture*. *Polymer*, 2005. **46**(26): p. 12662-12669.
 99. Manchineella, S., et al., *Surface-Functionalized Silk Fibroin Films as a Platform To Guide Neuron-like Differentiation of Human Mesenchymal Stem Cells*. *Acs Applied Materials & Interfaces*, 2016. **8**(35): p. 22849-22859.
 100. Azadi, M., A. Teimouri, and G. Mehranzadeh, *Preparation, characterization and biocompatible properties of beta-chitin/silk fibroin/nanohydroxyapatite composite scaffolds prepared using a freeze-drying method*. *Rsc Advances*, 2016. **6**(9): p. 7048-7060.
 101. Yan, L.P., et al., *Tumor Growth Suppression Induced by Biomimetic Silk Fibroin Hydrogels*. *Scientific Reports*, 2016. **6**.
 102. Bhattacharjee, P., et al., *Potential of inherent RGD containing silk fibroin-poly ((sic)-caprolactone) nanofibrous matrix for bone tissue engineering*. *Cell and Tissue Research*, 2016. **363**(2): p. 525-540.
 103. Sheikh, F.A., et al., *A novel approach to fabricate silk nanofibers containing hydroxyapatite nanoparticles using a three-way stopcock connector*. *Nanoscale Research Letters*, 2013. **8**.
 104. Tadepalli, S., et al., *Adsorption Behavior of Silk Fibroin on Amphiphilic Graphene Oxide*. *Acs Biomaterials Science & Engineering*, 2016. **2**(7): p. 1084-1092.
 105. Cai, Y.R., et al., *Silk fibroin membrane used for guided bone tissue regeneration*. *Materials Science & Engineering C-Materials for Biological Applications*, 2017. **70**: p. 148-154.
 106. Catto, V., et al., *Small diameter electrospun silk fibroin vascular grafts: Mechanical properties, in vitro biodegradability, and in vivo biocompatibility*. *Materials Science & Engineering C-Materials for Biological Applications*, 2015. **54**: p. 101-111.
 107. Tang, X., et al., *Bridging peripheral nerve defects with a tissue engineered nerve graft composed of an in vitro cultured nerve equivalent and a silk fibroin-based scaffold*. *Biomaterials*, 2012. **33**(15): p. 3860-3867.
 108. Hofmann, S., et al., *Silk fibroin as an organic polymer for controlled drug delivery*. *Journal of Controlled Release*, 2006. **111**(1-2): p. 219-227.
 109. Yang, Y., et al., *Development and evaluation of silk fibroin-based nerve grafts used for peripheral nerve regeneration*. *Biomaterials*, 2007. **28**(36): p. 5526-5535.
 110. Yang, M.C., et al., *The influence of rat mesenchymal stem cell CD44 surface markers on cell growth, fibronectin expression, and cardiomyogenic differentiation on silk fibroin - Hyaluronic acid cardiac patches*. *Biomaterials*, 2010. **31**(5): p. 854-862.
 111. Nampoothiri, K.M., N.R. Nair, and R.P. John, *An overview of the recent developments in polylactide (PLA) research*. *Bioresource technology*, 2010. **101**(22): p. 8493-8501.

112. Feng, S.-s. and G. Huang, *Effects of emulsifiers on the controlled release of paclitaxel (Taxol®) from nanospheres of biodegradable polymers*. Journal of Controlled Release, 2001. **71**(1): p. 53-69.
113. Asghari, F., et al., *Biodegradable and biocompatible polymers for tissue engineering application: a review*. Artificial cells, nanomedicine, and biotechnology, 2017. **45**(2): p. 185-192.
114. Rasal, R.M., A.V. Janorkar, and D.E. Hirt, *Poly (lactic acid) modifications*. Progress in polymer science, 2010. **35**(3): p. 338-356.
115. Dolynchuk, O., et al., *Reversible Shape-Memory Effect in Cross-Linked Linear Poly(ϵ -caprolactone) under Stress and Stress-Free Conditions*. Macromolecules, 2017. **50**(10): p. 3841-3854.
116. Gautam, S., A.K. Dinda, and N.C. Mishra, *Fabrication and characterization of PCL/gelatin composite nanofibrous scaffold for tissue engineering applications by electrospinning method*. Materials Science & Engineering C-Materials for Biological Applications, 2013. **33**(3): p. 1228-1235.
117. Jeon, H., H. Lee, and G. Kim, *A Surface-Modified Poly(ϵ -caprolactone) Scaffold Comprising Variable Nanosized Surface-Roughness Using a Plasma Treatment*. Tissue Engineering Part C-Methods, 2014. **20**(12): p. 951-963.
118. Wulf, K., et al., *Surface functionalization of poly(ϵ -caprolactone) improves its biocompatibility as scaffold material for bioartificial vessel prostheses*. J Biomed Mater Res B Appl Biomater, 2011. **98**(1): p. 89-100.
119. Recek, N., et al., *Cell Adhesion on Polycaprolactone Modified by Plasma Treatment*. International Journal of Polymer Science, 2016. **2016**: p. 9.
120. Lee, H., C.H. Jang, and G.H. Kim, *A polycaprolactone/silk-fibroin nanofibrous composite combined with human umbilical cord serum for subacute tympanic membrane perforation; an in vitro and in vivo study*. Journal of Materials Chemistry B, 2014. **2**(18): p. 2703-2713.
121. Anjum, F., et al., *Biocomposite nanofiber matrices to support ECM remodeling by human dermal progenitors and enhanced wound closure*. Scientific Reports, 2017. **7**.
122. Jiang, Y.C., et al., *Electrospun polycaprolactone/gelatin composites with enhanced cell-matrix interactions as blood vessel endothelial layer scaffolds*. Materials Science & Engineering C-Materials for Biological Applications, 2017. **71**: p. 901-908.
123. Srinivasa Reddy, C., et al., *Polycaprolactone/oligomer compound scaffolds for cardiac tissue engineering*. Journal of Biomedical Materials Research Part A, 2014. **102**(10): p. 3713-3725.
124. Thi Hiep, N., et al., *Biocompatibility of PCL/PLGA-BCP porous scaffold for bone tissue engineering applications*. Journal of Biomaterials Science, Polymer Edition, 2017. **28**(9): p. 864-878.
125. Barbarisi, M., et al., *Use of polycaprolactone (PCL) as scaffolds for the regeneration of nerve tissue*. Journal of Biomedical Materials Research Part A, 2015. **103**(5): p. 1755-1760.
126. Venugopal, J. and S. Ramakrishna, *Biocompatible nanofiber matrices for the engineering of a dermal substitute for skin regeneration*. Tissue Engineering, 2005. **11**(5-6): p. 847-854.

127. Zarekhalili, Z., et al., *Fabrication and characterization of PVA/Gum tragacanth/PCL hybrid nanofibrous scaffolds for skin substitutes*. International Journal of Biological Macromolecules, 2017. **94**: p. 679-690.
128. Chaudhari, A.A., et al., *Future Prospects for Scaffolding Methods and Biomaterials in Skin Tissue Engineering: A Review*. International Journal of Molecular Sciences, 2016. **17**(12).
129. Ranjbar-Mohammadi, M. and S.H. Bahrami, *Development of nanofibrous scaffolds containing gum tragacanth/poly (epsilon-caprolactone) for application as skin scaffolds*. Materials Science & Engineering C-Materials for Biological Applications, 2015. **48**: p. 71-79.
130. Ranjbar-Mohammadi, M., et al., *Antibacterial performance and in vivo diabetic wound healing of curcumin loaded gum tragacanth/poly(epsilon-caprolactone) electrospun nanofibers*. Materials Science & Engineering C-Materials for Biological Applications, 2016. **69**: p. 1183-1191.
131. Ahmed, S.M., et al., *Whey protein concentrate doped electrospun poly(epsilon-caprolactone) fibers for antibiotic release improvement*. Colloids and Surfaces B-Biointerfaces, 2016. **143**: p. 371-381.
132. Jérôme, C. and P. Lecomte, *Recent advances in the synthesis of aliphatic polyesters by ring-opening polymerization*. Advanced drug delivery reviews, 2008. **60**(9): p. 1056-1076.
133. Tao, X., *Smart fibres, fabrics and clothing: fundamentals and applications*. 2001: Elsevier.
134. Chattopadhyay, D.K. and K. Raju, *Structural engineering of polyurethane coatings for high performance applications*. Progress in polymer science, 2007. **32**(3): p. 352-418.
135. Mattmann, C., et al. *Recognizing upper body postures using textile strain sensors*. in *Wearable Computers, 2007 11th IEEE International Symposium on*. 2007. IEEE.
136. Abraham, T. and C. McMahan, *Thermoplastic Elastomers; Fundamentals and Applications*. Rubber Compounding; Chemistry and Applications, B. Rodgers, ed., Marcel Dekker, Inc., NY, 2004: p. 212.
137. ACQUARULO, L., C. O'Neil, and A. NILAJKAR, *Lubricious compounds for biomedical applications using hydrophilic polymers*. 2007, Google Patents.
138. Hong, Y., et al., *Tailoring the degradation kinetics of poly (ester carbonate urethane) urea thermoplastic elastomers for tissue engineering scaffolds*. Biomaterials, 2010. **31**(15): p. 4249-4258.
139. Chen, Q., S. Liang, and G.A. Thouas, *Elastomeric biomaterials for tissue engineering*. Progress in polymer science, 2013. **38**(3-4): p. 584-671.
140. Yilgor, I. and E. Yilgor, *Structure-Morphology-Property Behavior of Segmented Thermoplastic Polyurethanes and Polyureas Prepared without Chain Extenders*. Polymer Reviews, 2007. **47**(4): p. 487-510.
141. Oguz, O., et al., *Effect of soft segment molecular weight on the glass transition, crystallinity, molecular mobility and segmental dynamics of poly (ethylene oxide) based poly (urethane-urea) copolymers*. RSC Advances, 2017. **7**(65): p. 40745-40754.

142. Yilgör, I., E. Yilgör, and G.L. Wilkes, *Critical parameters in designing segmented polyurethanes and their effect on morphology and properties: A comprehensive review*. Polymer, 2015. **58**: p. A1-A36.
143. Barrioni, B.R., et al., *Synthesis and characterization of biodegradable polyurethane films based on HDI with hydrolyzable crosslinked bonds and a homogeneous structure for biomedical applications*. Materials Science and Engineering: C, 2015. **52**: p. 22-30.
144. Yilgör, E., et al., *Synthesis and structure-property behavior of polycaprolactone-polydimethylsiloxane-polycaprolactone triblock copolymers*. Polymer, 2016. **83**: p. 138-153.
145. Tatai, L., et al., *Thermoplastic biodegradable polyurethanes: the effect of chain extender structure on properties and in-vitro degradation*. Biomaterials, 2007. **28**(36): p. 5407-5417.
146. Wang, J., et al., *Synthesis and characterization of biodegradable polyurethanes based on L-cystine/cysteine and poly (ϵ -caprolactone)*. Journal of Applied Polymer Science, 2013. **128**(6): p. 4047-4057.
147. Bhowmick, A., et al., *Multifunctional zirconium oxide doped chitosan based hybrid nanocomposites as bone tissue engineering materials*. Carbohydrate Polymers, 2016. **151**: p. 879-888.
148. YILDIRIM, O., *Preparation and characterization of chitosan/calcium phosphate based composite biomaterials*. Materials Science and Engineering, 2004. **Master of Science**: p. 97.
149. Zhao, L. and J. Chang, *Preparation and characterization of macroporous chitosan/wollastonite composite scaffolds for tissue engineering*. Journal of Materials Science-Materials in Medicine, 2004. **15**(5): p. 625-629.
150. Noshi, T., et al., *Enhancement of the in vivo osteogenic potential of marrow/hydroxyapatite composites by bovine bone morphogenetic protein*. Journal of Biomedical Materials Research, 2000. **52**(4): p. 621-630.
151. Guo, B.L. and P.X. Ma, *Synthetic biodegradable functional polymers for tissue engineering: a brief review*. Science China-Chemistry, 2014. **57**(4): p. 490-500.
152. Carlos Peniche, et al., *Chitosan/hydroxyapatite-based composites*. Biotecnología Aplicada, 2010. **27**: p. 202-210.
153. Bressan, E., et al., *Biopolymers for Hard and Soft Engineered Tissues: Application in Odontoiatric and Plastic Surgery Field*. Polymers, 2011. **3**(1): p. 509-526.
154. Cai, Y.R., et al., *Role of hydroxyapatite nanoparticle size in bone cell proliferation*. Journal of Materials Chemistry, 2007. **17**(36): p. 3780-3787.
155. Liu, H.N. and T.J. Webster, *Mechanical properties of dispersed ceramic nanoparticles in polymer composites for orthopedic applications*. International Journal of Nanomedicine, 2010. **5**: p. 299-313.
156. Shen, D.Y., et al., *Structure and properties of polyacrylic acid modified hydroxyapatite/liquid crystal polymer composite*. Journal of Reinforced Plastics and Composites, 2011. **30**(13): p. 1155-1163.
157. Goonasekera, C.S., et al., *Dispersion of hydroxyapatite nanoparticles in solution and in polycaprolactone composite scaffolds*. Journal of Materials Chemistry B, 2016. **4**(3): p. 409-421.

158. Anwaar, M., E. Yilgör, and I. Yilgör. *Hydroxyapatite synthesis through sol-gel process and preparation of poly(lactic acid)/hydroxyapatite composites through electrospinning process*. in 15th AUTEX World Textile Conference. 2015. Romania: "Gheorghe Asachi" Technical University of Iasi, Romania, Faculty of Textiles, Leather and Industrial Management.
159. Ghosal, K., et al., *Structural and surface compatibility study of modified electrospun poly (ϵ -caprolactone)(pcl) composites for skin tissue engineering*. AAPS PharmSciTech, 2017. **18**(1): p. 72-81.
160. Goldstein, A.S., et al., *Effect of convection on osteoblastic cell growth and function in biodegradable polymer foam scaffolds*. Biomaterials, 2001. **22**(11): p. 1279-1288.
161. Istrate, O.M. and B. Chen, *Porous exfoliated poly (ϵ -caprolactone)/clay nanocomposites: Preparation, structure, and properties*. Journal of Applied Polymer Science, 2012. **125**(S1).
162. Jenkins, M., et al., *Characterisation of microcellular foams produced from semi-crystalline PCL using supercritical carbon dioxide*. European Polymer Journal, 2006. **42**(11): p. 3145-3151.
163. Salerno, A., et al., *Bio-safe fabrication of PLA scaffolds for bone tissue engineering by combining phase separation, porogen leaching and scCO₂ drying*. The Journal of Supercritical Fluids, 2015. **97**: p. 238-246.
164. Imhof, A. and D. Pine, *Ordered macroporous materials by emulsion templating*. Nature, 1997. **389**(6654): p. 948.
165. Önder, Ö.C., E. Yilgör, and I. Yilgör, *Fabrication of rigid poly (lactic acid) foams via thermally induced phase separation*. Polymer, 2016. **107**: p. 240-248.
166. Onder, O.C., E. Yilgor, and I. Yilgor, *Preparation of monolithic polycaprolactone foams with controlled morphology*. Polymer, 2017.
167. Sachlos, E. and J. Czernuszka, *Making tissue engineering scaffolds work. Review: the application of solid freeform fabrication technology to the production of tissue engineering scaffolds*. Eur Cell Mater, 2003. **5**(29): p. 39-40.
168. Leong, K., C. Cheah, and C. Chua, *Solid freeform fabrication of three-dimensional scaffolds for engineering replacement tissues and organs*. Biomaterials, 2003. **24**(13): p. 2363-2378.
169. Fina, F., et al., *Selective laser sintering (SLS) 3D printing of medicines*. International journal of pharmaceutics, 2017. **529**(1-2): p. 285-293.
170. Bose, S., S. Vahabzadeh, and A. Bandyopadhyay, *Bone tissue engineering using 3D printing*. Materials today, 2013. **16**(12): p. 496-504.
171. Gross, B.C., et al., *Evaluation of 3D printing and its potential impact on biotechnology and the chemical sciences*. 2014, ACS Publications.
172. Kao, C.-T., et al., *Poly (dopamine) coating of 3D printed poly (lactic acid) scaffolds for bone tissue engineering*. Materials Science and Engineering: C, 2015. **56**: p. 165-173.
173. Zein, I., et al., *Fused deposition modeling of novel scaffold architectures for tissue engineering applications*. Biomaterials, 2002. **23**(4): p. 1169-1185.
174. Hutmacher, D.W., et al., *Mechanical properties and cell cultural response of polycaprolactone scaffolds designed and fabricated via fused deposition modeling*. Journal of Biomedical Materials Research Part A, 2001. **55**(2): p. 203-216.

175. Senatov, F., et al., *Mechanical properties and shape memory effect of 3D-printed PLA-based porous scaffolds*. Journal of the mechanical behavior of biomedical materials, 2016. **57**: p. 139-148.
176. Hong, Y., et al., *A small diameter, fibrous vascular conduit generated from a poly (ester urethane) urea and phospholipid polymer blend*. Biomaterials, 2009. **30**(13): p. 2457-2467.
177. Luo, C., et al., *Electrospinning versus fibre production methods: from specifics to technological convergence*. Chemical Society Reviews, 2012. **41**(13): p. 4708-4735.
178. Soffer, L., et al., *Silk-based electrospun tubular scaffolds for tissue-engineered vascular grafts*. Journal of Biomaterials Science, Polymer Edition, 2008. **19**(5): p. 653-664.
179. Nazeer, M.A., E. Yilgör, and I. Yilgör. *Hydroxyapatite synthesis through sol-gel process and preparation of poly(lactic acid)/hydroxyapatite composites through electrospinning process*. in *15th AUTEX world textile conference, Bucharest, Romania*. 2015. AUTEX.
180. Zhang, Y., et al., *Preparation of core-shell structured PCL-r-gelatin bi-component nanofibers by coaxial electrospinning*. Chemistry of Materials, 2004. **16**(18): p. 3406-3409.
181. Sundararaghavan, H.G. and J.A. Burdick, *Gradients with depth in electrospun fibrous scaffolds for directed cell behavior*. Biomacromolecules, 2011. **12**(6): p. 2344-2350.
182. Hirata, Y., Y. Kanda, and N. Mataga, *Picosecond laser photolysis and transient photocurrent studies of the ionic dissociation mechanism of heteroexcimers: pyrene-N, N-dimethylaniline and pyrene-p-dicyanobenzene systems in polar solvents*. The Journal of Physical Chemistry, 1983. **87**(10): p. 1659-1662.
183. Hsu, C.M. and S. Shivkumar, *N, N-Dimethylformamide Additions to the Solution for the Electrospinning of Poly (ϵ -caprolactone) Nanofibers*. Macromolecular Materials and Engineering, 2004. **289**(4): p. 334-340.
184. Bilati, U., E. Allémann, and E. Doelker, *Development of a nanoprecipitation method intended for the entrapment of hydrophilic drugs into nanoparticles*. European Journal of Pharmaceutical Sciences, 2005. **24**(1): p. 67-75.
185. Nazeer, M.A., E. Yilgör, and I. Yilgör, *Intercalated chitosan/hydroxyapatite nanocomposites: Promising materials for bone tissue engineering applications*. Carbohydrate Polymers, 2017. **175**: p. 38-46.
186. Nazeer, M.A., E. Yilgör, and I. Yilgör. *Hydroxyapatite synthesis through sol-gel process and preparation of polylactic acid/hydroxyapatite composites through electrospinning process*. in *15th AUTEX world textile conference*. 2015. Bucharest/Romania.
187. Garskaite, E., et al., *Effect of processing conditions on the crystallinity and structure of carbonated calcium hydroxyapatite (CHAp)*. Crystengcomm, 2014. **16**(19): p. 3950-3959.
188. Catauro, M., et al., *Biological influence of Ca/P ratio on calcium phosphate coatings by sol-gel processing*. Materials Science & Engineering C-Materials for Biological Applications, 2016. **65**: p. 188-193.

189. Alobeedallah, H., et al., *Preparation of nanostructured hydroxyapatite in organic solvents for clinical applications*. Trends Biomater. Artif. Organs, 2011. **25**: p. 12-19.
190. Chen, K.H., et al., *Identification of monoclinic calcium pyrophosphate dihydrate and hydroxyapatite in human sclera using Raman microspectroscopy*. International Journal of Experimental Pathology, 2009. **90**(1): p. 74-78.
191. Sinko, K., *Influence of Chemical Conditions on the Nanoporous Structure of Silicate Aerogels*. Materials, 2010. **3**(1): p. 704-740.
192. Joshi, D.P., et al., *Effect of solvents on morphology, magnetic and dielectric properties of (α -Fe₂O₃@SiO₂) core-shell nanoparticles*. Heliyon, 2017. **3**(2): p. e00253.
193. Jouyban, A., S. Soltanpour, and H.K. Chan, *A simple relationship between dielectric constant of mixed solvents with solvent composition and temperature*. International Journal of Pharmaceutics, 2004. **269**(2): p. 353-360.
194. Streck, C. and R. Richert, *THE RELATION OF SOLVATOCHROMISM AND THERMOCHROMISM TO THE SOLVENT DIELECTRIC-CONSTANT - THE BASIS OF THE E(T) AND E'(T) POLARITY SCALES*. Berichte Der Bunsen-Gesellschaft-Physical Chemistry Chemical Physics, 1994. **98**(4): p. 619-625.
195. Jackelen, A.M.L., M. Jungbauer, and G.N. Glavee, *Nanoscale materials synthesis. 1. Solvent effects on hydridoborate reduction of copper ions*. Langmuir, 1999. **15**(7): p. 2322-2326.
196. Hu, A.M., et al., *Preparation of nanocrystals hydroxyapatite/TiO₂ compound by hydrothermal treatment*. Applied Catalysis B-Environmental, 2006. **63**(1-2): p. 41-44.
197. Asaoka, T., et al., *Development of bioactive porous alpha-TCP/HAp beads for bone tissue engineering*. Journal of Biomedical Materials Research Part A, 2013. **101**(11): p. 3295-3300.
198. Pighinelli, L. and M. Kucharska, *Chitosan-hydroxyapatite composites*. Carbohydrate Polymers, 2013. **93**(1): p. 256-262.
199. Mansur, H.S., et al., *Functionalized-chitosan/quantum dot nano-hybrids for nanomedicine applications: towards biolabeling and biosorbing phosphate metabolites*. Journal of Materials Chemistry B, 2013. **1**(12): p. 1696-1711.
200. Salimi, E. and J. Javadpour, *Synthesis and Characterization of Nanoporous Monetite Which Can Be Applicable for Drug Carrier*. Journal of Nanomaterials, 2012.
201. S.N. Danilchenko, et al., *Chitosan-hydroxyapatite composite biomaterials made by a one step co-precipitation method: preparation, characterization and in vivo tests*. JBPC, 2009. **9**: p. 119-126.
202. Tang, X., et al., *Influences of surface treatments with abrasive paper and sand-blasting on surface morphology, hydrophilicity, mineralization and osteoblasts behaviors of n-CS/PK composite*. Scientific Reports, 2017. **7**(1): p. 568.
203. Henkel, J., et al., *Bone Regeneration Based on Tissue Engineering Conceptions — A 21st Century Perspective*. Bone Research, 2013. **1**: p. 216.
204. Guo, Z., et al., *Characterization of biodegradable poly (lactic acid) porous scaffolds prepared using selective enzymatic degradation for tissue engineering*. RSC Advances, 2017. **7**(54): p. 34063-34070.

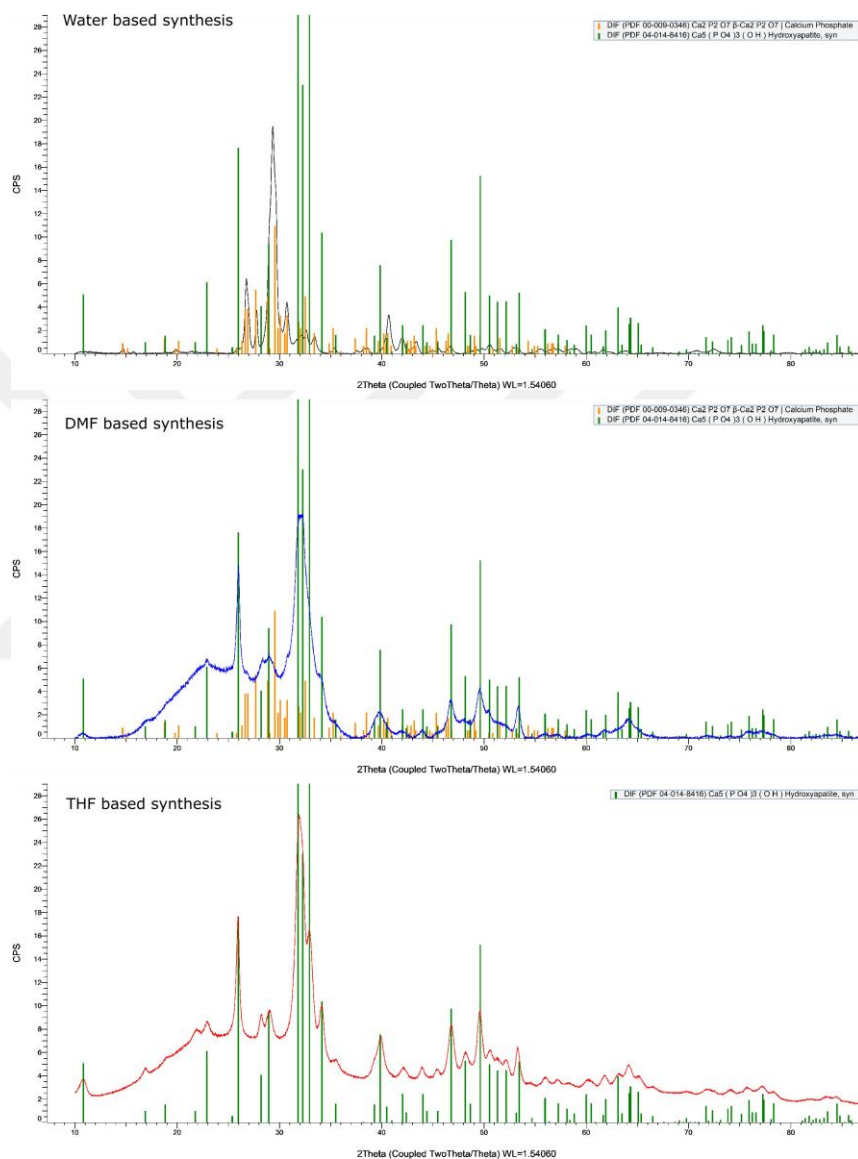
205. Rockwood, D.N., et al., *Materials fabrication from Bombyx mori silk fibroin*. Nature Protocols, 2011. **6**(10): p. 1612-1631.
206. Dou, H. and B.Q. Zuo, *Effect of sodium carbonate concentrations on the degumming and regeneration process of silk fibroin*. Journal of the Textile Institute, 2015. **106**(3): p. 311-319.
207. Bhardwaj, N., et al., *Milled non-mulberry silk fibroin microparticles as biomaterial for biomedical applications*. International Journal of Biological Macromolecules, 2015. **81**: p. 31-40.
208. Boulet-Audet, M., et al., *Attenuated total reflection infrared spectroscopy: An efficient technique to quantitatively determine the orientation and conformation of proteins in single silk fibers*. Applied Spectroscopy, 2008. **62**(9): p. 956-962.
209. Shen, G.T., et al., *Surface Modification and Characterisation of Silk Fibroin Fabric Produced by the Layer-by-Layer Self-Assembly of Multilayer Alginate/Regenerated Silk Fibroin*. Plos One, 2015. **10**(4).
210. Jin, H.J., et al., *Water-stable silk films with reduced beta-sheet content*. Advanced Functional Materials, 2005. **15**(8): p. 1241-1247.
211. Hu, X., D. Kaplan, and P. Cebe, *Determining beta-sheet crystallinity in fibrous proteins by thermal analysis and infrared spectroscopy*. Macromolecules, 2006. **39**(18): p. 6161-6170.
212. Tretinnikov, O.N. and Y. Tamada, *Influence of casting temperature on the near-surface structure and wettability of cast silk fibroin films*. Langmuir, 2001. **17**(23): p. 7406-7413.
213. Chen, X., et al., *Conformation transition kinetics of regenerated Bombyx mori silk fibroin membrane monitored by time-resolved FTIR spectroscopy*. Biophysical chemistry, 2001. **89**(1): p. 25-34.
214. Bradley, M., *Curve fitting in Raman and IR spectroscopy: basic theory of line shapes and applications*. Thermo Fisher Scientific, Madison, USA, Application Note, 2007. **50733**.
215. Rajkhowa, R., et al., *Structure and properties of biomedical films prepared from aqueous and acidic silk fibroin solutions*. Journal of Biomedical Materials Research Part A, 2011. **97**(1): p. 37-45.
216. Terada, D., et al., *The outermost surface properties of silk fibroin films reflect ethanol-treatment conditions used in biomaterial preparation*. Materials Science & Engineering C-Materials for Biological Applications, 2016. **58**: p. 119-126.
217. Bonazza, K., et al., *The fungal cerato-platanin protein EPL1 forms highly ordered layers at hydrophobic/hydrophilic interfaces*. Soft Matter, 2015. **11**(9): p. 1723-1732.
218. Food and D. Administration, *Guidance for Industry: Q3C—Tables and List*. USHaH Services (Ed.), Silver Spring, MD, 2012.
219. Lu, Q.A., et al., *Degradation Mechanism and Control of Silk Fibroin*. Biomacromolecules, 2011. **12**(4): p. 1080-1086.
220. Elzein, T., et al., *FTIR study of polycaprolactone chain organization at interfaces*. Journal of Colloid and Interface Science, 2004. **273**(2): p. 381-387.
221. Annaidh, A.N., et al., *Characterization of the anisotropic mechanical properties of excised human skin*. Journal of the mechanical behavior of biomedical materials, 2012. **5**(1): p. 139-148.

- 222. Marques, S.M. and J. da Silva, *Firefly Bioluminescence: A Mechanistic Approach of Luciferase Catalyzed Reactions*. Iubmb Life, 2009. **61**(1): p. 6-17.
- 223. Klinedinst, D.B., et al., *Structure—Property Behavior of New Segmented Polyurethanes and Polyureas Without Use of Chain Extenders*. Rubber chemistry and technology, 2005. **78**(5): p. 737-753.
- 224. Goetz, L.A., et al., *Superhydrophilic anti-fouling electrospun cellulose acetate membranes coated with chitin nanocrystals for water filtration*. Journal of Membrane Science, 2016. **510**: p. 238-248.
- 225. Horan, R.L., et al., *In vitro degradation of silk fibroin*. Biomaterials, 2005. **26**(17): p. 3385-3393.



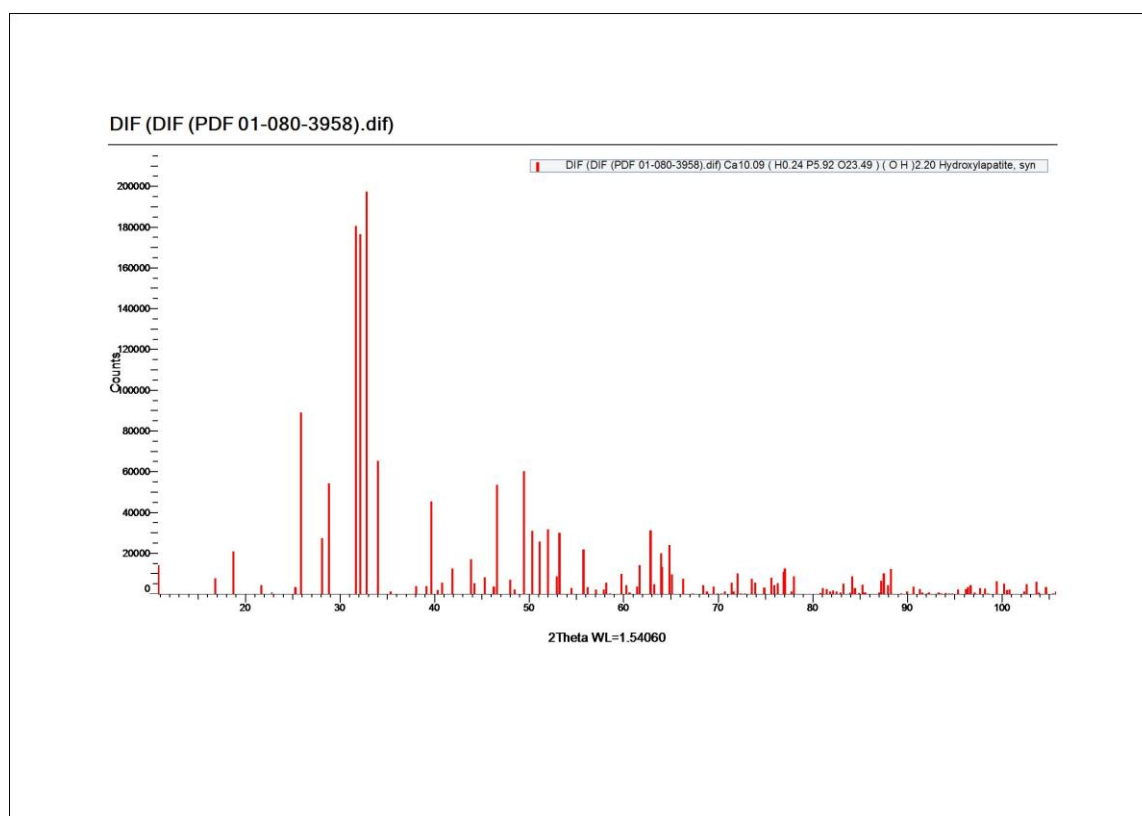
APPENDICES

Appendix-I



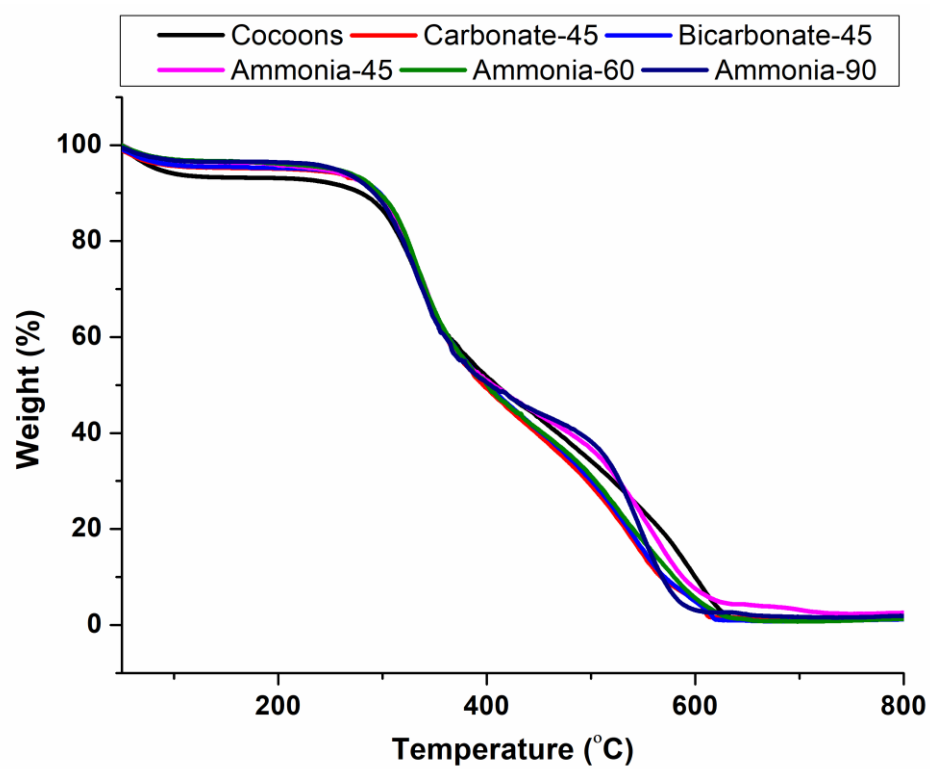
XRD analysis of CP synthesized through different solvent systems and comparison with database

Appendix-II



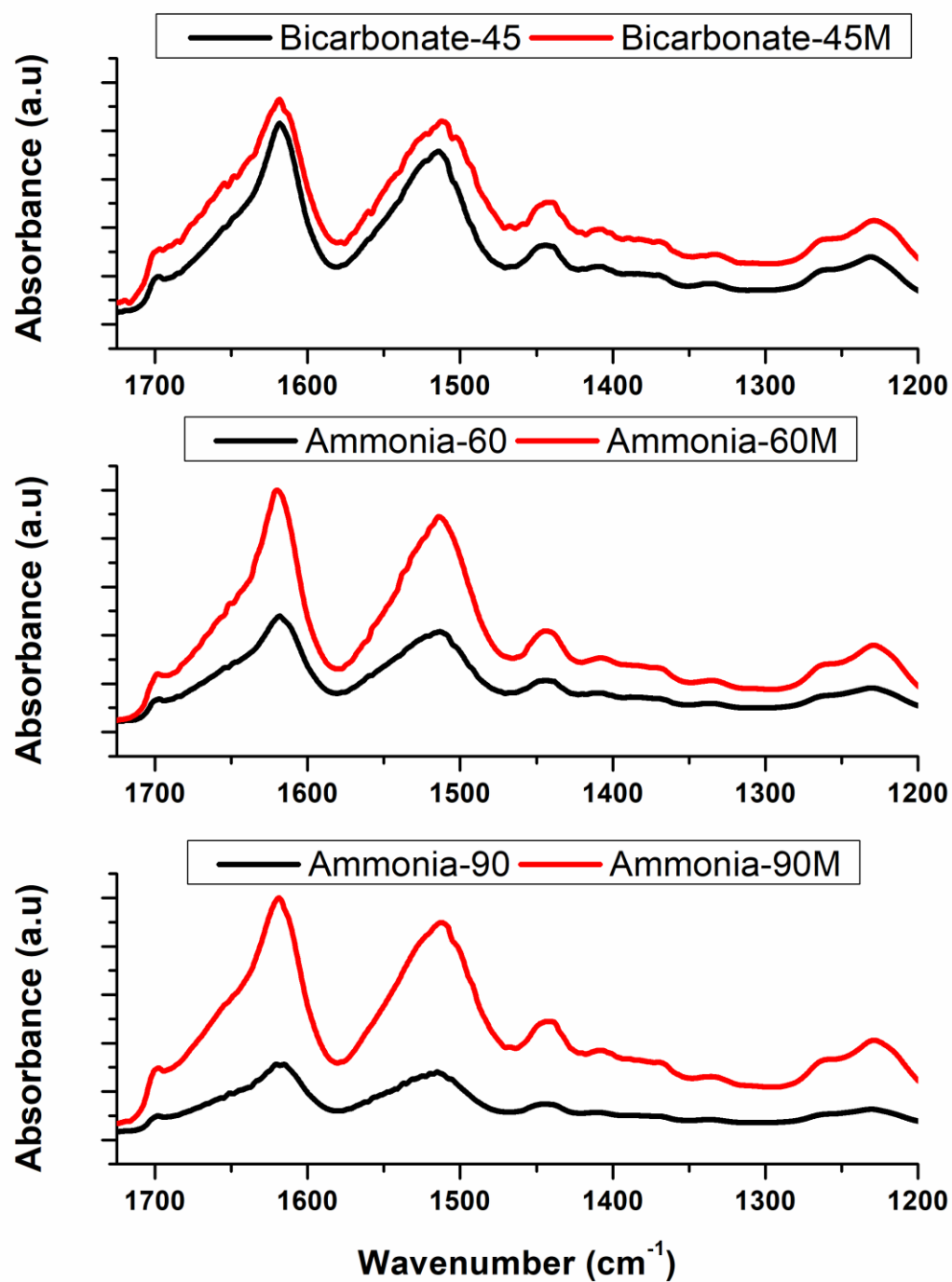
Standard XRD pattern of Hydroxyapatite (PDF 01-080-3958)

Appendix-III



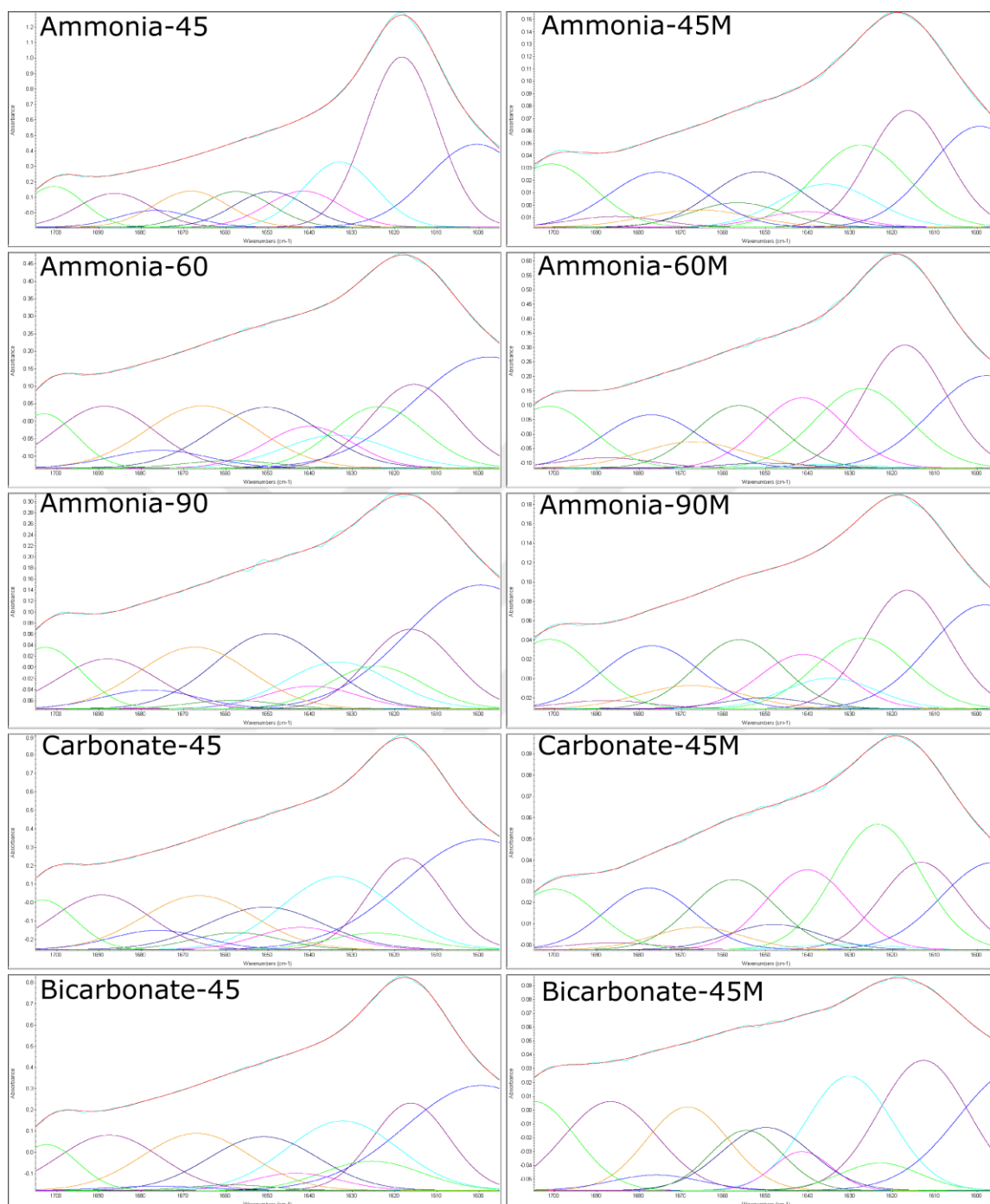
TGA analysis of cocoons and degummed fibers through different reagents and processing conditions

Appendix-IV



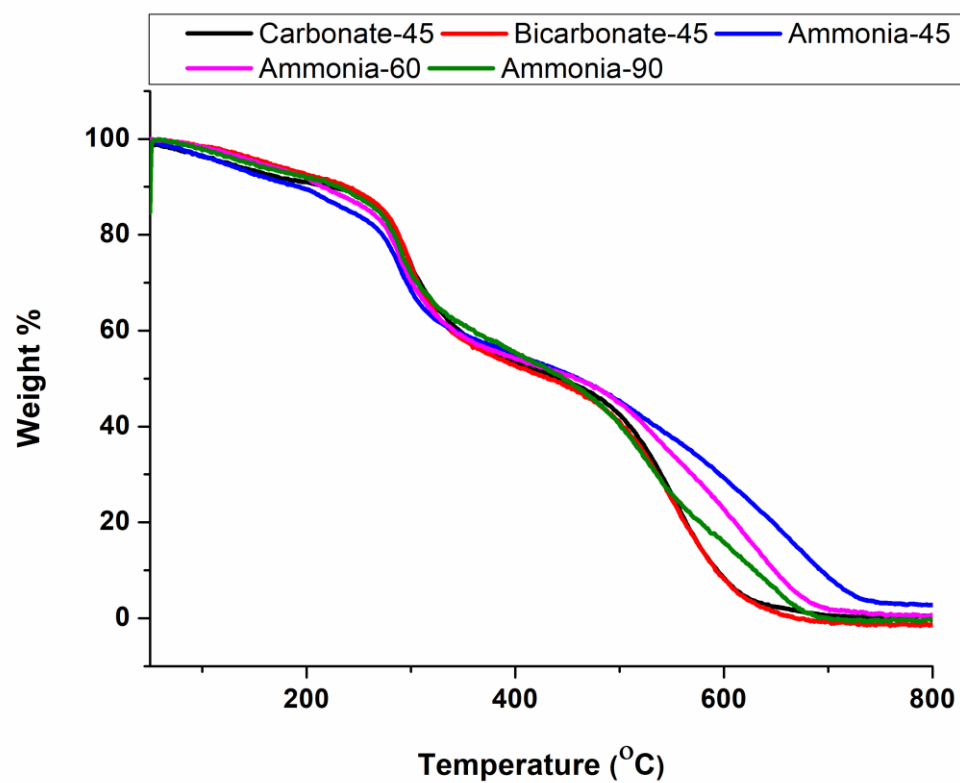
Comparison of ATR-FTIR spectra for silk fibroin films before and after methanol/water mixture treatment obtained through different processing routes

Appendix-V



Peaks resolved ATR-FTIR spectra of silk fibroin films obtained through different processing methods

Appendix-VI



TGA analysis of silk fibroin films obtained under different processing