

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE
ENGINEERING AND TECHNOLOGY

**PRODUCTION AND CHARACTERIZATION OF CHITOSAN-
HYDROXYAPATITE-FIBRINOGEN 3D SCAFFOLDS BY DIFFERENT
TECHNIQUES**

M.Sc. THESIS

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Department of Metallurgical and Materials Engineering

Materials Engineering Programme

MAY 2014

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Thesis Advisor: Prof. Dr. Gültekin GÖLLER

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**KİTOSAN-HİDROKSİAPATİT-FİBRİNOJEN ESASLI 3D YAPI
İSKELELERİNİN FARKLI YÖNTEMLE ÜRETİLMESİ VE
KARAKTERİZASYONU**

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To my lovely family,

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TABLE OF CONTENTS

	<u>Page</u>
FOREWORD	ix
TABLE OF CONTENTS.....	xi
ABBREVIATIONS.....	xiii
LIST OF TABLES.....	xv
LIST OF FIGURES.....	xvii
SUMMARY.....	xix
ÖZET.....	xxi
1. INTRODUCTION	1
1.1 Biomaterials.....	1
1.1.1 Chitosan.....	3
1.1.2 Hydroxyapatite	4
1.1.3 Fibrinogen	5
1.2 Scaffold	7
1.2.1 Scaffold production methods.....	7
1.3 Lyophilization.....	9
1.3.1 Principle of lyophilization	10
1.3.2 Mechanism of lyophilization.....	13
1.4 Electrospinning.....	14
1.4.1 Solution parameters	15
1.4.2 Processing parameters.....	16
1.4.3 Ambient parameters	16
2. MATERIALS AND METHODS	19
2.1 Materials and Laboratory Equipment	19
2.1.1 Equipment	19
2.1.2 Chemicals, Proteins and Buffers.....	19
2.2 Methods.....	19
2.2.1 The general outline of the experimental procedure	19
2.2.2 Preparation of hydroxyapatite by calcination and characterization.....	20
2.2.3 Preparation of the scaffolds	20
2.2.3.1 Preparation of CH-HA-Fib film.....	20
2.2.3.2 Preparation of CH-HA-Fib scaffold by lyophilization.....	21
2.2.3.3 Preparation of CH-HA-Fib scaffold by electrospinning	22
2.2.4 Characterization of scaffolds	22
2.2.4.1 Phase analysis	22
2.2.4.2 Molecular bond characterization analysis	22
2.2.4.3 Microstructure characterization	22
2.2.4.4 Swelling test.....	23
3. RESULTS AND DISCUSSION	25
3.1 Characterization of the hydroxyapatite powder	25
3.2 Characterization of Chitosan-Hydroxyapatite-Fibrinogen Scaffolds	26

3.2.1 Phase analysis.....	26
3.2.2 Molecular bond characterization.....	30
3.2.3 Microstructure characterization.....	34
3.2.4 Swelling tests	39
4. CONCLUSION	43
REFERENCES	45
APPENDICES	49
APPENDIX A	50
APPENDIX B.....	51
APPENDIX C.....	52
CURRICULUM VITAE.....	55

ABBREVIATIONS

HFIP	: 1,1,1,3,3,3 hexafluoro-2-propanol
AcOH	: Acetic acid
CH	: Chitosan
Fib	: Fibrinogen
HA	: Hydroxyapatite
PBS	: Phosphate Buffer Saline
TFA	: Trifluoroacetic acid

LIST OF TABLES

	<u>Page</u>
Table 1.1: Medical materials and their applications [1].....	2
Table 1.2: The main ions that exist in $M_{10}(ZrO_4)_6X_2$ [5].	4
Table 1.3: The major calcium phosphate forms [5].....	4
Table 1.4: Functions of extracellular matrix (ECM) in native tissues and scaffolds in engineered tissues [12].....	8
Table 1.5: Vapor pressure of ice [13].	12
Table 2.1: Different CH-HA and CH-HA-Fib films compositions.	20
Table 2.2: Different CH-HA and CH-HA-Fib scaffolds compositions.	21
Table C.0.1: Polymer solutions for film casting and lyophilization methods.....	53
Table C.0.2: Polymer solutions for electrospinning method.....	53

LIST OF FIGURES

	<u>Page</u>
Figure 1.1: Structure of chitosan monomers.	3
Figure 1.2: Structure of fibrinogen molecule [6].....	6
Figure 1.3: Schematic of fibrinogen molecule's structure.	6
Figure 1.4: Basic mechanism of tissue engineering [8].	7
Figure 1.5: Different techniques of scaffold production [11].	8
Figure 1.6: Water phase diagram [15].	10
Figure 1.7: Lyophilization cycle [14].	12
Figure 1.8: A schematic diagram of the major components of a lyophilizer.	13
Figure 1.9: Schematic of electrospinning A. Syringe with polymer solution, B. Taylor cone, C. Nanofibers jet, D. Collector plate, E. Power supply.	14
Figure 2.1: Lyophilizer, Alpha 1-2 LD Plus.	21
Figure 2.2: Field emission scanning electron microscope, JEOL JSM 7000F.	23
Figure 3.1: Particle size distribution of HA powder.	25
Figure 3.2: XRD pattern of pure HA powder.....	25
Figure 3.3: XRD pattern of pure low molecular weight chitosan.	26
Figure 3.4: XRD patterns of a)70CH30HA, b)60CH40HA, c)50CH50HA, d)40CH60HA, e)30CH70HA films.	27
Figure 3.5: XRD patterns of a) 30CH70HA b) 40CH60HA c) 50CH50HA d) 60CH40HA e) 70CH30HA lyophilized scaffolds.	28
Figure 3.6: XRD patterns of a)10CH1HA b) 10CH2HA c) 10CH3HA electrospinning scaffolds.....	29
Figure 3.7: FTIR spectrums of a) 30CH70HA, b) 40CH60HA, c) 50CH50HA, d) 60CH40HA, e) 70CH30HA films.	31
Figure 3.8: FTIR spectrum of a) pure HA, b) pure Fib., c) pure CH.....	31
Figure 3.9: FTIR spectrums of a) 30CH70HAFib, b) 40CH60HAFib, c) 50CH50HAFib, d) 60CH40HAFib, e) 70CH30HAFib films.	32
Figure 3.10: FTIR spectrums of lyophilized a) 30CH70HAFib,.....	33
Figure 3.11: FTIR spectrums of lyophilized a) 30CH70HA,.....	33
Figure 3.12: FTIR spectrums of electrospinning a)10CH1HA, b) 10CH2HA, c) 10CH3HA scaffolds.....	34
Figure 3.14: The SEM images of a) 30CH70HA, b) 40CH60HA, c) 50CH50HA, d) 60CH4HA, e) 70CH30HA, f) 30CH70HAFib, g) 40CH60HAFib, ...	36
Figure 3.13: The SEM images of a) 30CH70HA, b) 40CH60HA, c) 50CH50HA, d) 60CH4HA, e) 70CH30HA, f) 30CH70HAFib, g) 40CH60HAFib, ...	37
Figure 3.15: The SEM images of nanofibers a) 6% CH in TFA, b) 8% CH in TFA, c) 10% CH in TFA, d) 10% CH and 1% HA in TFA, ...	38
Figure 3.16: Swelling ratios of films and lyphlized scaffolds without fibrinogen. ..	40
Figure 3.17: Swelling ratios of films and lyphlized scaffolds with fibrinogen.	40

PRODUCTION AND CHARACTERIZATION OF CHITOSAN-HYDROXYAPATITE-FIBRINOGEN 3D SCAFFOLDS BY THREE DIFFERENT TECHNIQUES

SUMMARY

People may have tissue or organ loss as a result of accidents or diseases. Difficult and troublesome processes, such as organ transplantation, may be required for the treatment of such damages. However, different techniques have been developed to produce artificial tissues and organs through the advanced technology and life sciences. One of the main techniques to develop these artificial body parts is scaffold production. Not only the technique, but also choosing of the true material, which depends on the structure and location of the tissue is an important step for production of scaffolds. Although ceramics, metals and composites are used a lot as biomaterials, polymer materials are preferred due to the properties such as easy to produce, variable techniques of production, easy to shape.

Chitosan, a linear polysaccharide, is widely used as biomaterials. The chitosan is formed by copolymerization of N-acetyl-D-glucosamine and D-glucosamine. The main reasons of the having a widely application range of chitosan are biocompatibility, biodegradable, anti-toxic, anti-tumor, anti-microbial and wound healing properties. Hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_2\text{OH}_2$, is the key inorganic compound of the hard tissue of vertebra. The hydroxyapatite powder for experiments was obtained from bovine bone by calcination at 850°C for 4 hours. Fibrinogen is a protein, which is responsible for blood coagulation, cell-cell and cell-tissue interactions, inflammatory response and wound healing.

The aim of this study is production of scaffolds by lyophilization and electrospinning techniques besides polymer films for using in hard tissue applications. On this purpose, the specimens, which contain chitosan-hydroxyapatite and chitosan-hydroxyapatite-fibrinogen compounds in different concentrations were produced.

After the fabrication of the scaffolds, phase, molecular bond characterization and microstructure characterization were conducted. According to XRD patterns, the polymer films and lyophilized scaffolds have crystal structure because of hydroxyapatite. The peaks match the pure hydroxyapatite XRD pattern. Moreover, the interactions between hydroxyapatite, chitosan and fibrinogen were determined by molecular bond characterization tests. The specific peak at 1010 cm^{-1} show the hydroxyapatite presence. The peaks around $1500\text{-}1600$ and $3000\text{-}3500\text{ cm}^{-1}$ show that the interactions between chitosan, hydroxyapatite and fibrinogen. Another characterized property of the productions was microstructure. The porous structure of lyophilized productions and nanofibers structure of electrospinning productions was observed after microstructure characterization, which is very important to cell proliferation by obtaining larger area and nutrient flow. The absorption of water, which is already a property of chitosan, was improved by addition of fibrinogen.

In conclusion, three-dimensional scaffolds, containing chitosan, hydroxyapatite and fibrinogen and being used in hard tissue applications, were produced successfully and the interactions between materials were conducted by characterization tests. The

SEM images show that the microstructure of the scaffolds, which are produced by , is damaged by increasing of HA concentration. On the other hand, molecular interactions between materials present except changing the intensity of the bands due to the change of the concentrations. The swelling ratios of the products did not change because of the production technique, but did increase due to the addition of fibrinogen.

KİTOSAN-HİDROKSİAPATİT-FİBRİNOJEN ESASLI 3D YAPI İSKELELERİNİN ÜÇ FARKLI YÖNTEMLE ÜRETİMİ VE KARAKTERİZASYONU

ÖZET

Çeşitli sebeplerle ortaya çıkan kazalar ve hastalıklar sonucu insanlar doku ve organ kaybı yaşamaktadırlar. Bu tür hasarların tedavisi için de organ nakli gibi zor ve zahmetli yöntemler gerekebilmektedir. Fakat gelişen teknoloji ve yaşam bilimleri sayesinde yapay doku ve organ üretimi için çeşitli yöntemler geliştirilmiştir. Bu yöntemlerin en başında da yapı iskeleleri gelmektedir. Yapı iskelelerinin üretimde farklı biyomalzemeler kullanılmaktadır. Malzeme seçimi yapı iskelesinin nerede ve hangi amaçla kullanılacağına bağlı olarak değişir. Fakat polimer malzemeler, hem kolay üretilmesi hem de üretim çeşitlerinin çokluğu, malzemenin kolay şekil alabilmesi gibi özelliklerinden dolayı doku mühendisliği alanında oldukça fazla tercih edilmektedir.

Kitosan, biyomalzeme olarak doku mühendisliği alanında oldukça sık kullanılan lineer, doğal bir polisakkarittir. Yine doğal bir polisakkarit olan kitinin deasetilasyonu sonucu, N-asetil-D-glikozamin ve D-glikozamin monomerlerinden oluşur. Kitosanın bu kadar çok kullanılmasının sebebi; biyoyumlu, biyobozunur, toksik olmayan, antitümör, antimikrobiyal ve yara iyileştirici olmasıdır.

Moleküler içeriğine ve Ca/P molar oranına göre kalsiyum fosfat bileşikleri farklı alanlarda kullanılabilirler. Hidroksiapatit ise 1,67 Ca/P oranına ve $\text{Ca}_{10}(\text{PO}_4)_6\text{OH}_2$ formülüne sahip, omurgalı canlılardaki sert dokuların temel bileşenlerinden biridir. Kemik gibi bulunduğu yapılara sert bir karakteri kazandırır. Deneyler boyunca kullanılan hidroksiapatit, 850°C’de 4 saat boyunca kalsine edilen sıgır kemiğinden elde edilmiştir.

Fibrinojen ise kanda ve hücre dışı sıvıda bulunan çözülebilir bir proteindir. Bu proteinin en büyük özelliği kanama başladığı zaman fiberler oluşturarak kanın pıhtılaşmasında rol almasıdır. Kanın pıhtılaşma mekanizmasının yanı sıra, hücreler arası ve hücre-doku arası etkileşimler, inflamatuvar cevap, yara iyileşmesi, yeni doku oluşumu gibi görevleri de üstlenmektedir. Protein, iki tane dışta D ve bir tane merkezde E domaininden oluşmaktadır. Bu domainler birbirlerine $\text{A}\alpha$, $\text{B}\beta$ ve γ polipeptidleri ile bağlanmaktadır.

Bu çalışmanın amacı sert doku uygulamalarında kullanılabilecek yapı iskelelerini film üretiminin yanı sıra liyofilizasyon ve elektroegirme yöntemleri ile üretmektir. Bu amaç doğrultusunda farklı bileşimlerde kitosan-hidroksiapatit ve kitosan-hidroksiapatit-fibrinojen bileşimli numuneler üretilmiştir. Çalışmada yapı iskelesi üretimi için üç farklı yöntem kullanılmıştır.

Bunlardan birincisi film dökmedir, %2 (h/h) asidik asit içerisinde oluşturulan ve daha sonra NaOH ile pH’ı 7,4’e ayarlanan polimer çözeltisinin bir petri kabına dökülmesi ve oda sıcaklığında kurutulması sonucunda polimer filmler elde edilmiştir.

Bir diğer yöntem olarak da liyofilizasyondan faydalanılmıştır. Liyofilizasyon yönteminin temel prensibi, suyun dondurulması sonucu oluşan buz kristallerinin

süblimleştirilmesi sonucu malzemeyi kurutmaktır. Bu yöntemin genel kullanım amacı gıdaların ve çabuk bozulabilecek organik malzemelerin daha uzun süre saklanmasını sağlamak olsa da doku mühendisliğinde de por oluşturmak amaçlı da oldukça fazla kullanılan bir yöntemdir. Bu amaçla %2 (h/h) astik asit içerisinde oluşturulan ve daha sonra NaOH ile pH'ı 7,4'e ayarlanan polimer çözeltisi hazırlanmıştır. Hazırlanan çözeltiler -20°C'de 24 saat boyunca dondurulmuş ve daha sonra 6 gün boyunca liyofilize edilmiştir.

Son üretim yöntemi olan elektroegirmede ise amaç polimer çözeltisine yüksek voltaj uygulayıp toplayıcı plaka ile iğne ucu arasında oluşacak manyetik alan sayesinde polimer solüsyonun saçılarak fiber oluşturmasını sağlamaktır. Burada üretim sistemine etki eden bir çok parametre vardır. Bunların başlıcaları; voltaj değeri, pompalama hızı ve toplayıcı plakanın uzaklığıdır. Kitosan-hidroksiapatit esaslı malzemelerin elektroegirme yöntemi ile üretilmesi içinse çözücü olarak saf halde trifloraasetik asit kullanılmıştır. Kitosanın bu çözücü içinde çözünmesinin ardından eklenen hidroksiapatit ile süspansiyon hazırlanmış ve 17 kV voltaja maruz bırakılmıştır. Bu işlem sırasında polimer çözeltisinin pompalama hızı 0,4 ml/s ve iğne ucu işle toplayıcı plaka arasındaki mesafe de 10 cm olarak belirlenmiştir.

Üretim sonucunda faz analizi, moleküler bağ karakterizasyonu ve mikroyapı karakterizasyonu uygulanmıştır.

Faz analizi ile birlikte amorf yapıda olan kitosan ve fibrinojen, hidroksiapatit ile liyofilizasyon ve film üretimi sonucu kristalize bir yapı kazandığı gözlenmiştir. 10-80° 2θ değerleri arasında 2°/dk hız ile yapılan faz analizi sonucu saf hidroksiapatitin 9-0432 kart numaralı hidroksiapatit olduğu sonucu elde edilmiştir. Aynı zamanda saf hidroksiapatit tozlarına uygulanan XRD sonrasında β-TCP'ye ait pikler gözlenmemiştir. Bu da üretilen hidroksiapatitin biyolojik olarak türetildiğinin bir başka göstergesidir. Film ve liyofilizasyon ile üretilen yapı iskelelerindeki piklerin de yine hidroksiapatite ait karakteristik pikler olduğu gözlenmiştir. Yapılan işlemler sırasında kitosan kristal bir yapı göstermemiştir. Fakat kitosan miktarı arttıkça pik şiddetleri düşmüştür. Bunun yanında elektroegirme sonucunda malzeme amorf yapısını korumuştur.

Yapılan karakterizasyon çalışmaları sonucunda liyofilizasyon ile porlu, üç boyutlu ve elektroegirme ile nanolif yapıda yapı iskeleleri başarıyla üretildiği görülmüştür.

Moleküler bağ karakterizasyonu ilk olarak başlangıç tozlarına yapılmıştır. Yapılan analizler sonucunda tozların saf olduğu anlaşılmıştır. Üretilen film ve yapı iskelelerine yapılan moleküler bağ karakterizasyonu sonucu 3000-3500 cm⁻¹ civarında -OH ve -CH₃ fonksiyonel gruplarına ait bantlar gözlenirken, 1027 cm⁻¹'de de PO₄⁻³ fonksiyonel gurubuna ait pikler göze çarpmıştır. 1600-1500 cm⁻¹ aralığında da amit I, amit II ve amit III fonksiyonel gruplarının varlığı tespit edilmiştir. Bu gruplar hem fibrinojenden hem de kitosandan kaynaklıdır.

Üretilen filmlerin ve doku iskelelerinin mikroyapıları taramalı elektron mikroskopu ile incelenmiştir. Polimer filmlerin yüzey morfolojisi incelendiğinde hidroksiapatit ilavesi ile birlikte yüzeyin porlu bir yapı kazandığı gözlenmiştir. Fakat fibrinojen ilavesi ile bu porlu yapı ortadan kalkmış ve yeni oluşan moleküler etkileşimlerin yardımıyla oldukça porsuz bir yüzey morfolojisi gözlenmiştir. Tüm bunların yanında yüzeyde hidroksiapatit tanecikleri de gözlenmiştir. Amaçlandığı gibi liyofilizasyon sonucu porlu, elektroegirme sonucu da fiber yapı başarıyla elde edilmiştir. Liyofilizasyon üretimi ile porların yapısı hidroksiapatit miktarının artması ile değişmiştir. Ayrıca fibrinojen ilavesi de por boyutunun büyümesini sağlamıştır. Nanolif yapısı da hidroksiapatit katkısıyla incelenmiştir.

Su hapsetme özelliđi olan kitosanın bu karakteri fibrinojen ilavesi ile arttırılmıřtır fakat hidroksiapatit katkısının nasıl bir etkisi olduđu incelenmemiřtir. Bunun yanında üretim yöntemi de su hapsetme özelliđine belirgin bir avantaj ya da dezavantaj sađlamamıřtır.

Sonuç olarak sert doku uygulamalarında kullanılacak olan kitosan-fibrinojen-hidroksiapatit bileřimli yapı iskelesi üretimi başarıyla sonuçlanmış ve sonrasında yapılan karakterizasyon çalıřmalarıyla örneklerin özellikleri incelenmiřtir. Beklenildiđi üzere film üretimi ile porsuz, tamamen katı bir yapı elde edilirken, liyofilizasyon ile porlu, elektroēirme ile de lifli yapılar elde edilmiřtir. Üretim teknikleri kıyaslandıđında ise kristalizasyon ve moleküler bađ karakterizasyonu ađısından film ve liyofilizasyon üretimi ile ilgili belirgin bir fark yokken elektroēirmede faz analizi sonuçları hidroksikarbona apatit piklerine rastlanmamıřtır.

1. INTRODUCTION

In the last couple of decades, biomedical scientists have been studying different materials more and more for their biological use and benefit and the efforts to find novel materials have resulted in the discovery of numerous valuable materials. The chemical and physical structures for many of these materials have been determined and improved to be used as biomaterials. Metals, ceramics, polymers and composites are basic types of biomaterials. According to their properties, these materials are used to regenerate damaged or non-functional body parts.

Biomaterials are biodegradable, bioactive, biocompatible, anti-tumor, anti-microbial matter that interacts with biological systems without foreign body reaction. According to the area of using, the properties can be modified. When the biomaterial and the tissue are in contact, no toxic product should be released. Especially blood-contacting biomaterials are exposed to enzymes that damage the surface structure. Thus, these biomaterials have to be more resistant not to lose their performance.

The aim of our study is to produce a novel composite of chitosan, hydroxyapatite and fibrinogen as a 3D scaffold. Within the framework of this aim, chitosan, hydroxyapatite and fibrinogen 3D scaffolds are fabricated in different concentrations by film casting, and electrospinning techniques and these products are characterized.

1.1 Biomaterials

Biomaterials are natural or synthetic materials that interact with biological systems or used in medical devices and they could be in solid or liquid form. Biomaterials have existed for almost half a century and their use have spread to diverse fields such as medicine, biology, chemistry and material science. There are different types of matter that could be used as biomaterials; metals, ceramics and polymers. Each material has various properties; therefore, they can be used in different applications according to their properties (Table 1.1).

Using of biomaterials dates far back into the ancient times. For instance, artificial teeth, ear, eyes and nose were found mummies from Egypt [2]. Unfortunately, these medical implants were designed with limited consideration of the interaction

between the material and the biological systems. The first modern medical implant was developed in 1949 by a British ophthalmologist Harold Ridley. He observed the Spitfire fighter pilots and worked on lenses. Subsequent, Harold Ridley continued his work on intraocular lenses (IOLs) while Charnley developed the hip implant and Vorhees designed the vascular graft, which was followed by the invention of the kidney dialysis and the ball and cage heart valve [3].

Table 1.1: Medical materials and their applications [1].

Material	Applicaitons	Material	Applicaitons
Metals and alloys		Polymers	
316L Satinless steel	Fracture fixation, stents, surgical instruments	Polyethylene	Joint replacement
CP-Ti, Ti-Al-V, Ti-Al-Nb, Ti-13Nb-13Zr, Ti-Mo-Zr-Fe	Bone and joint replacement, fracture fixation, dental implants, pacemaker encapsulation	Polypropylene	Sutures
Co-Cr-Mo, Cr-Ni-Cr-Mo	Dental implants, bone and joint replacement, dental restorations, heart valves	PET	Sutures, vascular prosthesis
Ni-Ti	Bone plates, stents, orthodontic wires	Polyamides	Sutures
Gold alloys	Dental restorations	PTFE	Soft-tissue augmentation, vascular prostheses
Silver products	Antibacterial agents	Polyesters	Vascular prostheses, drug-delivery systems
Hg-Ag-Sn amalgam	Dental restorations	Polyurethanes	Blood-contacting devices
Ceramics and glasses		Composites	
Alumina	Joint replacement, dental implants	BIS-GMA-quartz/silica filler	Dental restorations
Zirconia	Joint replacement	PMMA-glass fillers	Dental restorations (dental cements)
Calcium phosphates	Bone repair and augmentation, surface coatings on metals		
Bioactive glasses	Bone replacement		

1.1.1 Chitosan

Chitosan (CH) is a linear, cationic polysaccharide, which is formed by copolymerization of D-glucosamine and N-acetyl-D-glucosamine residues with β 1-4 glucosidic linkage and it is also formed by deacetylation of N-acetyl-D-glucosamine in chitin, a natural polymer (Figure 1.1). Chitosan is a biocompatible, biodegradable, non-toxic, anti-tumor, wound-healing and antimicrobial polysaccharide. Due to these properties, chitosan is widely-used in tissue engineering and regenerative medicine [4].

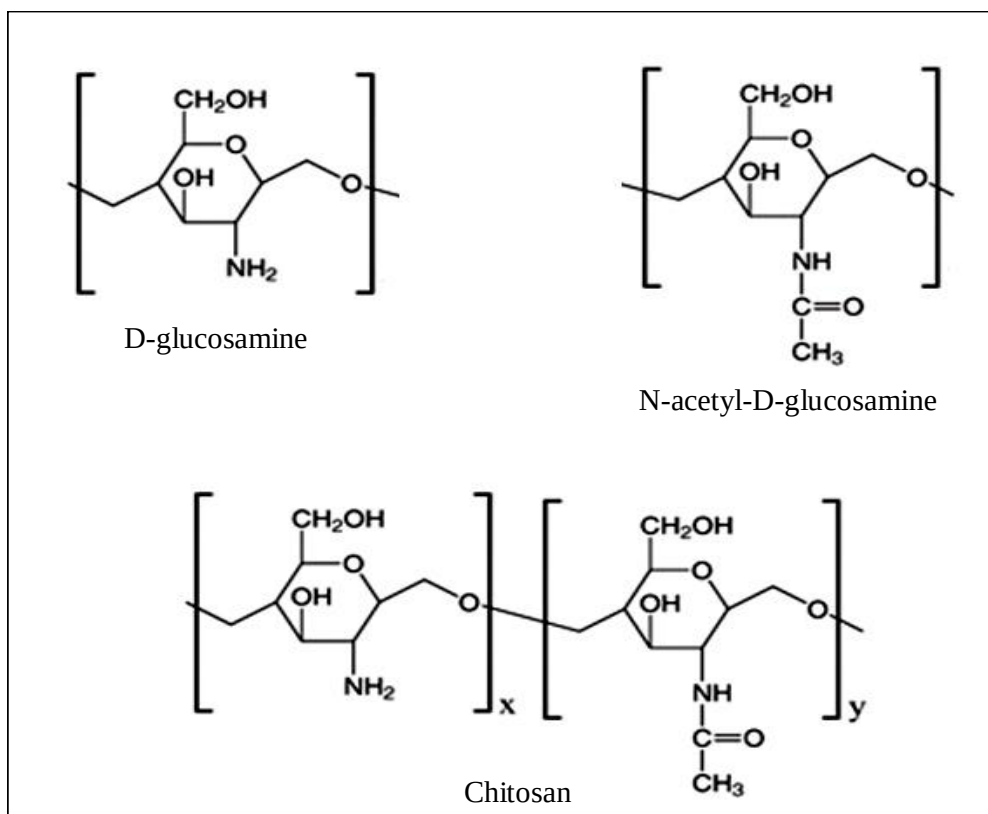


Figure 1.1: Structure of chitosan monomers.

Chitosan can dissolve in aqueous acidic media because about 50% of N-acetyl-D-glucosamine in chitin has already been deacetylated. The -NH_2 functional group on C-2 position on the repeat unit of D-glucosamine provides the solubilization by protonation, which gives polyelectrolyte character to the polysaccharide. This unique character makes chitosan pseudonatural cationic polymer. A re-adjusting of the pH to 7.4, the natural pH, causes flocculation of chitosan [4].

1.1.2 Hydroxyapatite

The general formula of apatite is $M_{10}(ZO_4)_6X_2$ or $M_5(ZO_4)_3X$. “Apatite” (απαταω), which means deceit in Greek, is the origin of the word “apatite”. The reason for this naming is the fact that the mineral has different colors because of the different crystal habits and thus was mistaken for precious minerals [5].

Each $M_5(ZO_4)_3X$ component has a different solid system and the elements in the formula could be found in Table 1.2. In some cases M or X can be absent.

Table 1.2: The main ions that exist in $M_{10}(ZO_4)_6X_2$ [5].

	Type of ions
M	Ca^{2+} , Mg^{2+} , Sr^{2+} , Ba^{2+} , Mn^{2+} , Fe^{2+} , Zn^{2+} , Cd^{2+} , Pb^{2+} , Na^+ , K^+ , Al^{3+}
ZO₄	PO_4^{3-} , AsO_4^{3-} , VO_4^{3-} , CO_3^{2-} , SO_4^{2-} , SiO_4^{4-}
X₂	F_2^- , Cl_2^- , Br_2^- , O_2^- , $(OH)_2$, CO_3^{2-}

The calcium phosphate apatite, where M is Ca^{2+} and ZO_4 is PO_4^{3-} , is the most common form of the apatite family. If X is fluorine it is called fluorapatite. When the formula becomes $Ca_{10}(PO_4)_6OH_2$ the compound is called hydroxyapatite, HA, which is the key inorganic component of the hard tissue of vertebrae. The major calcium phosphate types are given in Table 1.3.

Table 1.3: The major calcium phosphate forms [5].

Name	Formula	Ca/P
Monocalcium phosphate anhydrous (MCPA)	$Ca(HPO_4)_2$	0.50
Monocalcium phosphate monohydrate (MCPM)	$Ca(HPO_4)_2 \cdot H_2O$	0.50
Dicalcium phosphate anhydrous (DCPA•monetite)	$CaHPO_4$	1.00
Dicalcium phosphate dihydrate (DCPD•brushite)	$CaHPO_4 \cdot 2H_2O$	1.00
Alpha tricalcium phosphate (α-TCP)	$Ca_3(PO_4)_2$	1.50
Beta tricalcium phosphate (β-TCP)	$Ca_3(PO_4)_2$	1.50
Hydroxyapatite (HA)	$Ca_{10}(PO_4)_6OH_2$	1.67
Tetracalcium phosphate	$Ca_4(PO_4)_2O$	2.00

Hydroxyapatite is the most stable form of calcium phosphate family at physiological pH. Besides HA, β -TCP is a hypothermic phase and it is also used in bioactive ceramics instead of HA due to its higher absorption ability. Otherwise, rather than pure HA or pure β -TCP, the composite of these two forms of calcium phosphate, called biphasic calcium phosphate (BCP), is preferred. On the other hand, amorphous calcium phosphate (ACP) has priority in some cases as a result of its bioresorbable property. At higher temperatures β -TCP transforms into α -TCP, which is stable between 1180 and 1430 °C. Furthermore, MCPM, DCPA, DCPD and TeCP are other forms of calcium phosphate.

1.1.3 Fibrinogen

One of the most important actors of the blood clotting mechanism is fibrinogen (fib), which provides cellular and matrix interactions, fibrinolysis, inflammatory response, wound healing and neoplasia. Fibrinogen by itself or fibrin form of the fibrinogen can perform these purposes according its form [6].

As shown in Figure 1.2, fibrinogen has two D outer domains and one E central domain, which is connected to each D domain by a coiled-coiled segment. Two sets of three polypeptide chains named $A\alpha$, $B\beta$ and γ , which are connected to each other and to the E domain from their N-terminus by five symmetrical sulfide bridges, generate the fibrinogen molecule [6].

While the $A\alpha$ -chain consists of 610 and $B\beta$ -chain consists of 461 residues, the major γ -chain form γA has 411 residues. On the other hand γ' chain is an alternative form of the γ chain, which is formed by alternative processing of the mRNA transcript, with an addition of a unique sequence of twenty anionic amino acids. Approximately 8% of the total fibrinogen in the blood carries heterodimeric (γ/γ') γ -chain while less than 1% of the fibrinogen carries homodimeric (γ'/γ') γ -chain [7].

Fibrinogen is a thrombin substrate with two active sites in the N-terminus, namely, fibrinopeptide A (FPA) and fibrinopeptide B (FPB), which are the sequences for initiation of fibrin assembly (Figure 1.3). By the cleavage of thrombin the two short peptides (FPA and FPB), the N-termini of the $A\alpha$ and $B\beta$ chains, fibrin formation from fibrinogen is started. The new N-terminal sequences of both chains located on the E domain, named 'knobs', can interact with the D domain of the fibrin. By the

help of factor XIIIa the fibrin polymers cross-link between amino acids, glutamine and lysine [8].

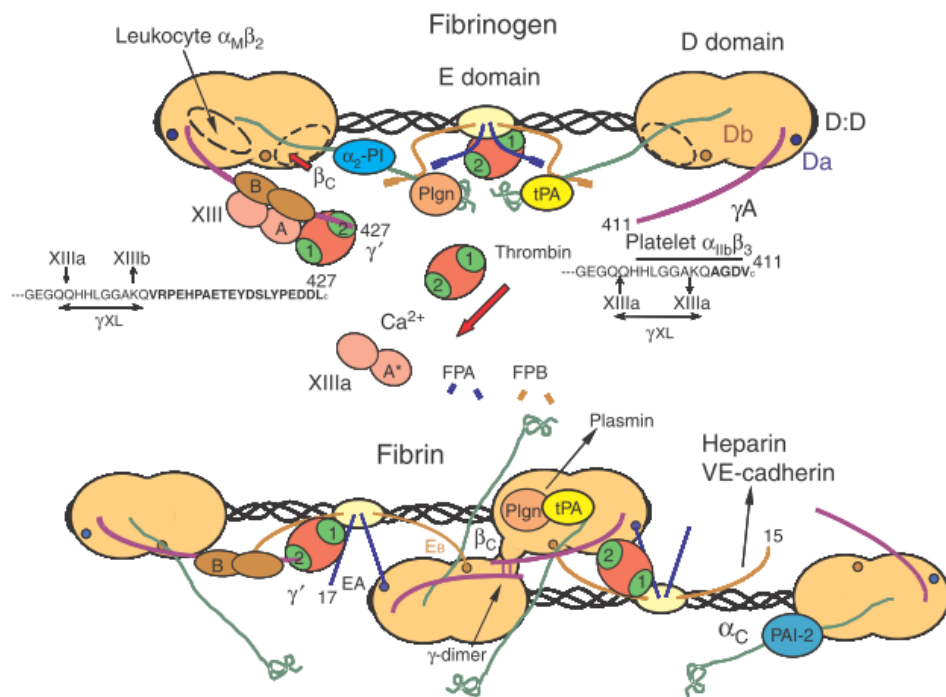


Figure 1.2: Structure of fibrinogen molecule [6].

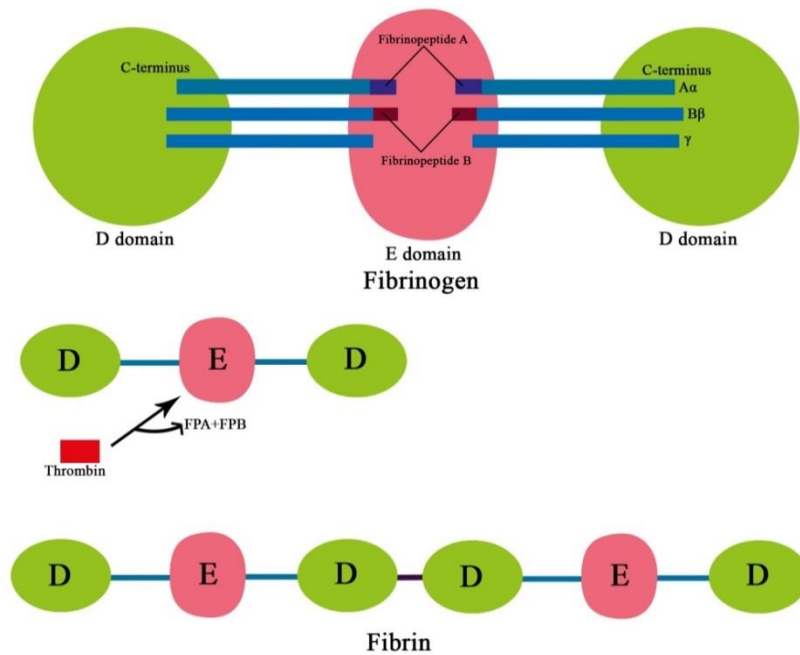


Figure 1.3: Schematic of fibrinogen molecule's structure.

1.2 Scaffold

Tissue engineering, an interdisciplinary area, requires knowledge from both the life sciences and engineering in order to produce artificial tissues and even organs in the laboratory. Production of new biological materials, improvement and development of their properties as biological body parts are the responsibility of tissue engineering. For this purpose, the most common procedure to fabricate these products is scaffolding (Figure 1.4).

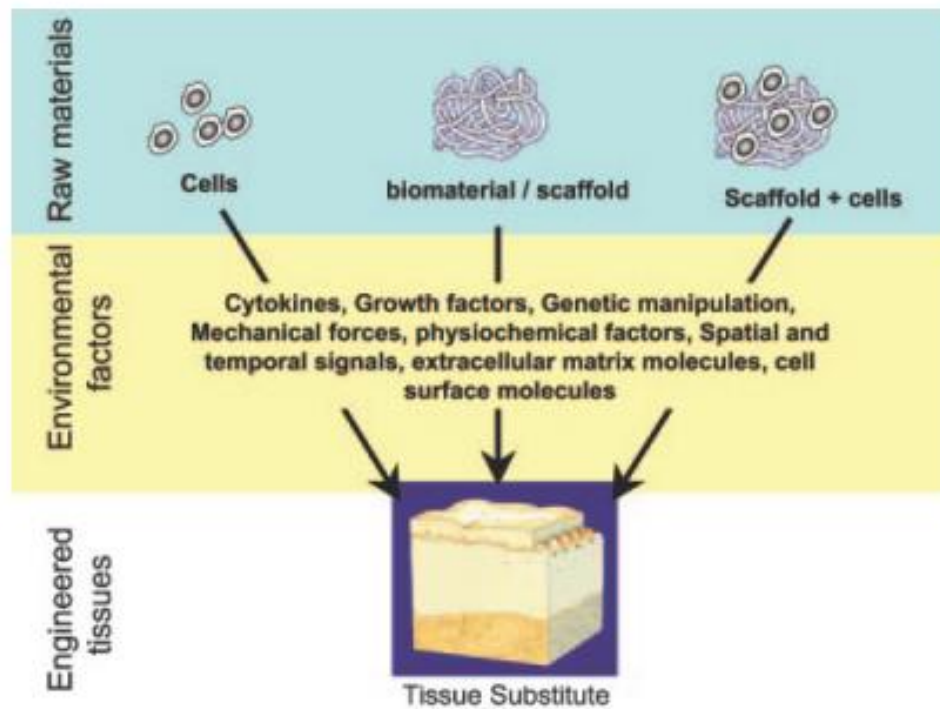


Figure 1.4: Basic mechanism of tissue engineering [8].

A scaffold which can be either natural or synthetic materials is produced as a temporary support for the cells that will be placed in the body. Cells are seeded to the scaffold and colonize it until they are compatible and viable in the body. There are a number of key points to design a scaffold. Firstly, the body part's physical and chemical properties, where scaffold will be placed, should be well known for choosing suitable material and shape of the scaffold [9-12].

1.2.1 Scaffold production methods

The scaffold, which mimics extracellular matrix (ECM) both physically and chemically, serves as a framework to support cell proliferation on it and makes the cells migrate to the defected area from surrounding tissues (Table 1.4) [12].

Table 1.4: Functions of extracellular matrix (ECM) in native tissues and scaffolds in engineered tissues [12].

Functions of ECM in native tissues	Analogous functions of scaffolds in engineered tissues	Architectural, biological, and mechanical features of scaffolds
1. Provides structural support for cells to reside	Provides structural support for exogenously applied cells to attach, grow, migrate and differentiate in vitro and in vivo	Biomaterials with binding sites for cells; porous structure with interconnectivity for cell migration and for nutrients diffusion; temporary resistance to biodegradation upon implantation
2. Contributes to the mechanical properties of tissues	Provides the shape and mechanical stability to the tissue defect and gives the rigidity and stiffness to the engineered tissues	Biomaterials with sufficient mechanical properties filling up the void space of the defect and simulating that of the native tissue
3. Provides bioactive cues for cells to respond to their microenvironment	Interacts with cells actively to facilitate activities such as proliferation and differentiation	Biological cues such as cell-adhesive binding sites; physical cues such as surface topography
4. Acts as the reservoirs of growth factors and potentiates their actions	Serves as delivery vehicle and reservoir for exogenously applied growth-stimulating factors	Microstructures and other matrix factors retaining bioactive agents in scaffold
5. Provides a flexible physical environment to allow remodeling in response to tissue dynamic processes such as wound healing	Provides a void volume for vascularization and new tissue formation during remodeling	Porous microstructures for nutrients and metabolites diffusion; matrix design with controllable degradation mechanisms and rates; biomaterials and their degraded products with acceptable tissue compatibility

According to the Table 4, there are four important features and functions to produce a scaffold; architecture, cyto-tissue compatibility, bioactivity and mechanical property.

In addition, there are four different approaches to produce a scaffold in tissue engineering (Figure 1.5) [12].

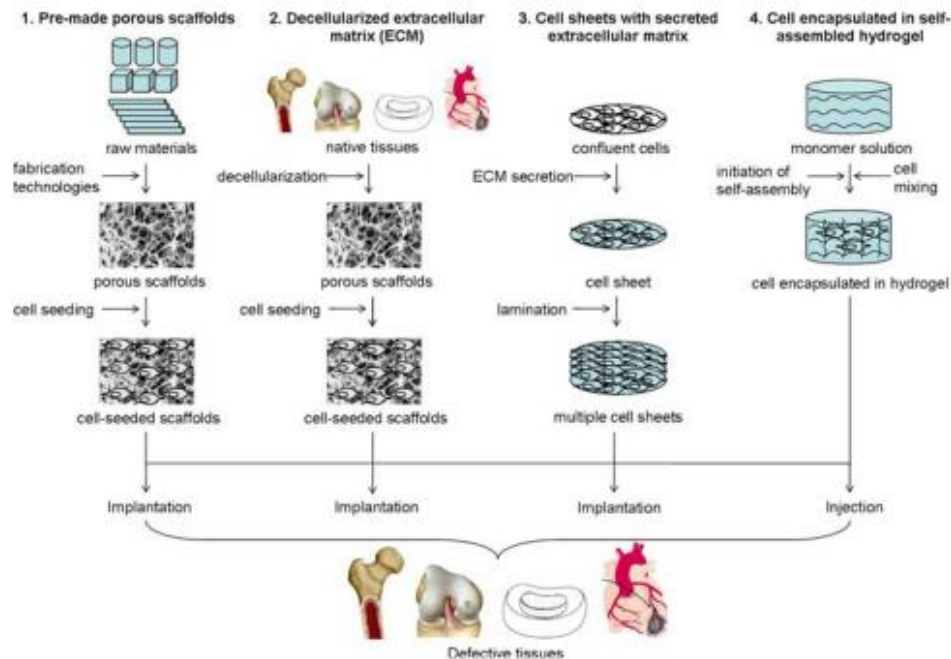


Figure 1.5: Different techniques of scaffold production [11].

- **Pre-made porous scaffolds:** This kind of scaffold can be fabricated by either synthetic or natural biomaterials. With different techniques, solid, porous or fibrous scaffolds are produced and cells are seeded on. These products have a very large using area including soft and hard tissue applications. However, cell seeding process may be a disadvantage due to lowering the cell viability.
- **Decellularized extracellular matrix:** An allogenic or xenogenic tissue is decellularized not to damage the extracellular matrix. Rest of the tissue without the cells are seeded with healthy cells and implanted to the defected area. These products are very biocompatible compared to the others and fix the exact shape of the tissue. On the other hand, difficulties during the decellularization process may cause some immune problems and unhealthy cells may remain.
- **Cell sheets with secreted ECM:** The cell layers are produced without enzymatic treatments, which produce their own extracellular matrix. The cells are cultured on a thermo-responsive polymer that helps the cell harvesting without enzymes and the need for alignment of the cells. This process can be repeated to produce multilayer cell sheets to get thicker matrix.
- **Cells encapsulated in self-assembled hydrogel:** Cells are added into the hydrogel solution and self-assembly is performed with treatment with UV, different pH or temperature. There are both cells and hydrogels in the final product, as if cells are in suspension. These scaffolds can be used in soft tissue applications.

1.3 Lyophilization

All organic matter includes water, which is one of the main solvents and provides organisms a suitable environment to survive. On the other hand organic materials may need to be stored for a long time, and during this time water should be removed not to provide the microorganisms an appropriate setting to live [13].

Firstly, people used brine method by increasing salt concentration of the water. Another method was drying food under the sun, and then freezing as an alternative method has been developed. None of these methods provided a long term protection until lyophilization technique [14].

Besides, lyophilization is used to produce scaffold as tissue engineering applications. During lyophilization process, pores are formed due to the water sublimation. Sublimated water transports to the surface and leave the solution thus there will be

pores instead of water molecules. The cell adhesion and migration will increase as an advantage of porous structure, because the pores and interconnections between pores will provide a larger area and good nutrient flow [14].

1.3.1 Principle of lyophilization

According to the lyophilization technique, materials are dehydrated by sublimation. Two parameters are very important during the process; temperature and pressure. The freeze drying process consists of three stages: prefreezing, primary drying and secondary drying [14].

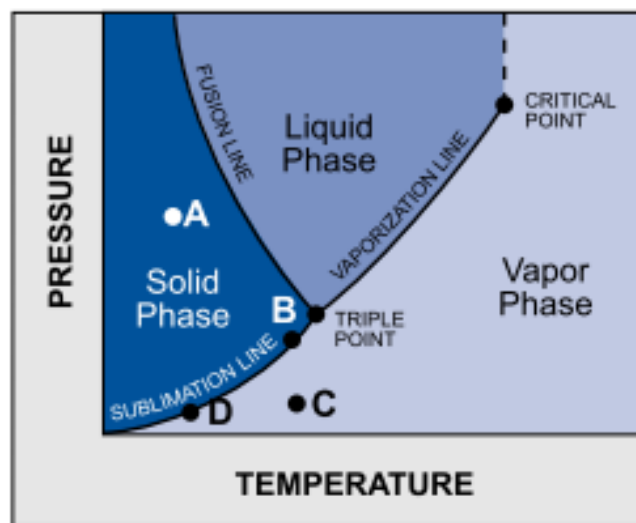


Figure 1.6: Water phase diagram [15].

Prefreezing: Before the sublimation process the specimen should be fully frozen. The freezing speed and final temperature of the specimen affect the results of process. Small ice crystals are formed after rapid cooling and this obstructs drying stage. To produce larger ice crystals and prevent restrictive channels, the cooling speed must be slow. There are two ways to freeze;

According to the first method, products are frozen under triple point (Point B, Figure 1.6). During cooling process, water molecules separate from suspension/solution, which becomes more concentrated due to the formation of ice crystals. It is very important that the specimen must be under triple point to resume a successful lyophilization process.

The second type of freezing is glass formation. The specimen is cooled under glass transition temperature and glass formation occurs. Drying of this kind of specimen is

extremely difficult. Some products like aqueous sucrose solutions can undergo structural deformation such as collapse, thus the freezing temperature must be below -32°C .

Primary drying: There are three important factors during the primary drying process: temperature, pressure and energy.

The main focus of the primary drying is removing water molecules by sublimation and generates a dry, structurally intact product. To form this product, the first thing to do is to remove the water molecules by sublimation. The water molecules must sublime and form vapor to leave the product by pressure difference. Hence, the product must be warmer than the ice condenser which provides difference between vapor pressure of product and vapor pressure of ice condenser (Table 1.5). To start the lyophilization process, the product's temperature must be below triple or glass transition temperature and the product must be subjected to reduced pressure. There must be a vacuum system, which supplies pressure below Point C and assists water molecules to move from high pressure to low pressure.

The condensing system collects the condensable gases and vacuum pump collects non-condensable gases. Pressure difference between product and condenser makes the transportation of the gases to the condenser possible. As it is mentioned before, the temperature difference between the condenser and the product (the condenser is cooler than the product) originates from pressure difference.

Besides temperature and pressure, the energy, which is needed for transportation of the water molecules, is one of the key points of the lyophilization process. Each water molecule needs energy to break the bonds, move to the surface and leave the product. This energy is provided by heat. The heat can be obtained in different ways like a shelf as a drying tray or from environment. However, the temperature of the product must not exceed the triple point or collapse temperature.

After a water molecule breaks from the material it needs to transport to the surface and leave the material. The main driving force of the movement of the water molecule is free space of the vacuum. By sublimation of water molecules, a dry fragment occurs on the frozen wet sample's surface. Gradually, this dry part gets thicker and the distance taken by water molecules gets longer [13-15].

Table 1.5: Vapor pressure of ice [13].

Temperature (°C)	P (mm Hg)	Temperature (°C)	P (mm Hg)
-80	0,0004	-30	0,285
-70	0,0019	-25	0,476
-60	0,0080	-20	0,776
-50	0,0295	-15	1,241
-45	0,054	-10	1,950
-40	0,096	-5	3,013
-35	0,164	0	4,579

Secondary drying: Even though all ice crystals leave the product after primary drying, bound moisture is still present in the product. To remove the rest of the moisture the drying process should continue under a warmer temperature named as isothermal desorption. Success at final drying depends on temperature of the product, which is higher than environment's [13-15].

Whole lyophilization cycle is shown in Figure 1.7.

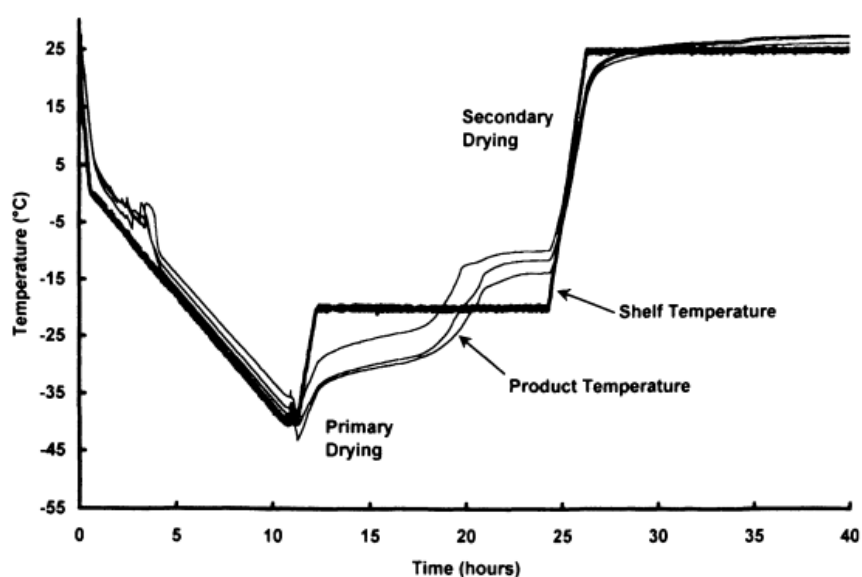


Figure 1.7: Lyophilization cycle [14].

1.3.2 Mechanism of lyophilization

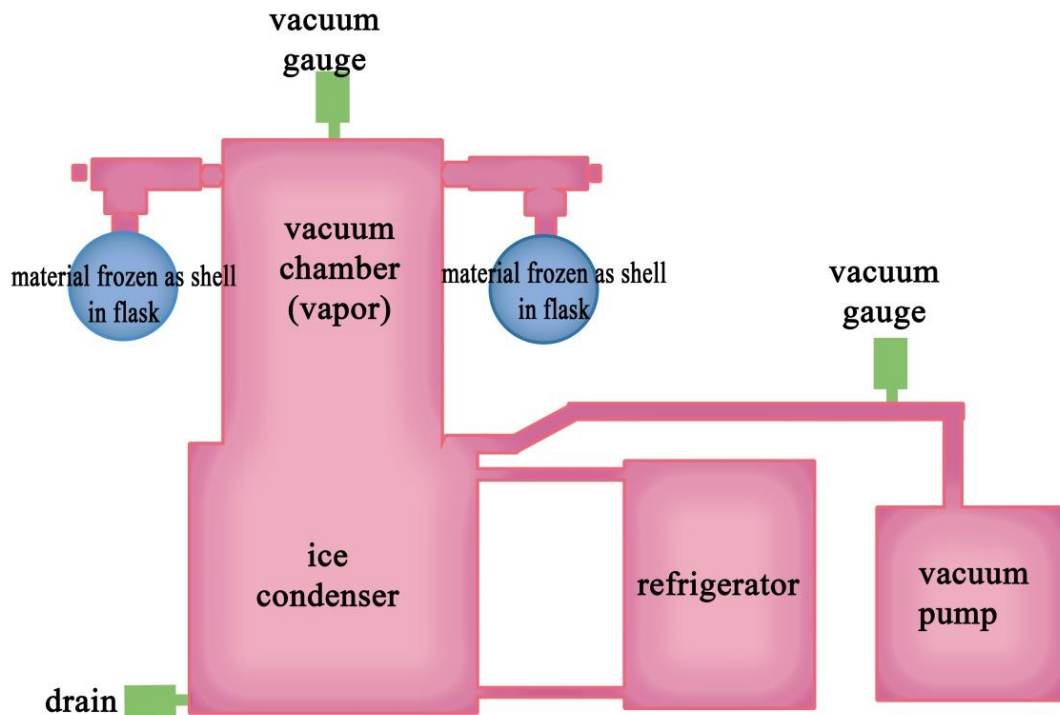


Figure 1.8: A schematic diagram of the major components of a lyophilizer.

A schematic diagram of the major components of a lyophilizer is shown in Figure 1.8. One of the main parts of the lyophilizer is vacuum chamber, which includes hollow shelves with heat transferred fluid circulating in them. This system helps the temperature calibration between -50°C and $+50^{\circ}\text{C}$. These shelves supply the energy for sublimation of the ice crystals, which is approximately 670 cal/g. Ice condenser collects water vapor by condensation at about -70°C . An inducing force, which is low pressure, is needed for transportation of gases. The vacuum pump maintains the system pressure at 50 mTorr or less [14].

Another inducing force of transportation of the water molecules is pressure difference between the product and environment as the result of temperature difference. A few temperature sensors are placed to determine the product's temperature during the drying process. As a result; the microstructure of the material and a high specific surface area are conserved after drying. When all ice crystals have sublimed, the heat is not needed anymore and the product temperature increases to the shelf temperature [15].

1.4 Electrospinning

Electrospinning is a simple and efficient method to produce nano or micro scale polymer fibers as scaffolds in similar structure with extracellular matrix. During electrospinning process, the polymer solution is pumped at a constant rate through a syringe and subjected to high-voltage. The high-voltage supplies an electric field and makes the polymer spin in this electric field. The spinning polymer attaches to the collecting plate and the fibers are generated by help of the volatility of the solvent (Figure 1.9) [16].

In detail, by turning on the electric power, an electric field is created between the needle and metallic collecting plate. At the same time, a polymer droplet is formed on the needle by pumping mechanism, this droplet shape transforms to conic shape named Taylor cone due to the electric field. By the time Taylor cone becomes more instable and finally organic solvent evaporates and the polymer forms into fiber jets which are collected on the metal collector plate. The final product is a fibrous structure that has various applications in several areas ranging from drug delivery to tissue engineering [16].

The architecture of final product, which is fabricated by electrospinning is similar to extracellular matrix. The nutrient or body fluids can be transported through the fibers and the chemical concentrations of the fibers can effect cellular response and new tissue formation. The cell adhesion and migration increase by both fibrous and porous structure of the electrospinning scaffolds [16].

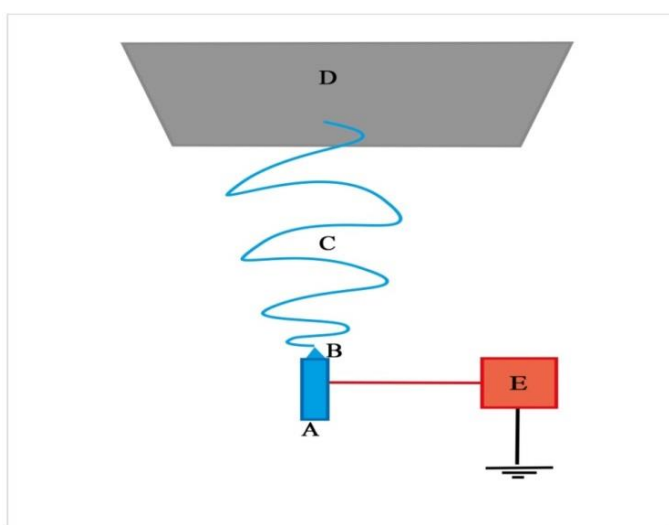


Figure 1.9: Schematic of electrospinning A. Syringe with polymer solution, B. Taylor cone, C. Nanofibers jet, D. Collector plate, E. Power supply.

1.4.1 Solution parameters

The character of the polymer solution determines the morphology of the final product of the electrospinning. There are five main parameters as polymer's properties that affect the electrospinning process and fiber formation [17];

1. *Concentration* is the main parameter of the nanofiber formation, which determines final product's shape as fibers or beads. For low concentrations of the polymer solution the procedure ends up with electrospray instead of electrospinning due to the low viscosity and high surface tension of the solution. By increasing the concentration of the solution, a fiber and bead mixed structure is formed and at the suitable concentration fine nanofibers are generated. If the concentration of the solution is too high, helix-shaped micro ribbons are developed.

In addition, increasing the concentration enlarges the diameter of the fibers between the critical concentration limits.

2. *Molecular weight* of the polymer affects the morphology of the fibers efficiently due to viscosity of the solution. If two solutions were prepared at the same concentration with low and high molecular weight polymers, beads would form with low molecular weight polymer while fibers would form with high molecular weight polymer. As a result, increasing molecular weight of the polymer simplifies the fiber formation. However, there is a limit for molecular weight as if it is too high, the micro-ribbons are formed instead of fibers.

Nevertheless, molecular weight is not the essential parameter when sufficient intramolecular interactions are obtained by oligomers.

3. Another key point of the fiber formation is *viscosity* of the solution. In very low viscosity, the fibers are not generated. Besides, in high viscosity, ejection of the polymer is too hard for jet formation. The suitable viscosity for fine fiber formation is adjusted using concentration of polymer solution and polymeric molecular weight. Additionally, the viscosity affects the surface tension that creates beads or beaded fibers.

4. *Surface tension* is the energy required for increasing the surface area of the liquid and results imbalance between intermolecular forces [18]. The electrical force must overcome the surface tension of the polymer solution to form the electrically charged

polymer jet [19]. In brief, when all the conditions are optimized, surface tension determines upper and lower boundaries of the process.

5. Even if all four of the conditions are obtained; the electrospinning process cannot work without a conductive polymer solution. The *conductivity* of the solution depends on the polymer, solvent type and the salt. The ionic salts or organic solvents can provide conductivity.

1.4.2 Processing parameters

Beside the solution properties, the electrospinning process conditions are very effective on the nanofiber formation. Those parameters could be listed as [17]:

1. The formation of the jet from Taylor cone depends on the applied *voltage* magnitude that must be higher than threshold voltage. However, the certain effect of the voltage on the fiber diameter is not known yet. Several groups claim that, the increase of the voltage increases the fiber diameter. In contrast, several groups suggest that high voltage increases the electrostatic repulsive force on the jet that decreases the fiber diameter. Additionally, according to other groups, high voltage induces bead formation during the process.

2. *Flow rate* determines the time for polarization and fiber formation of the polymer. When flow rate is high, bead fibers with thick diameters are generated, on the other hand low flow rate provides thin, smooth fibers due to short drying time and low stretching force.

3. The *collectors* should be conductive to collect charged fibers. Usually, Al foil is used as collector but it is difficult to remove the fibers from the foil. On this purpose, different collector types have been developed such as wire mesh, pin, grids, parallel or gridded bar and so on.

4. *The distance between the collector and the tip of the needle* is also effective on the fiber morphology. The optimum distance provides enough time for solidification of the polymer and prevents bead formation.

1.4.3 Ambient parameters

Environment may affect the fiber formation during the electrospinning process [17].

1. There is an inverse relationship between *temperature* and viscosity, if temperature increases, the viscosity decreases. Thus the increase of the temperature decreases the diameter of the fibers.
2. While low *humidity* dries the solvent totally by increasing evaporation velocity, high humidity enlarges fiber diameter due to decreasing stretching force by neutralization of the charges on the jet.

2. MATERIALS AND METHODS

This study includes two major parts. In the first part, chitosan-hydroxyapatite-fibrinogen scaffolds were prepared by film casting, and electrospinning techniques. In order to do that, chitosan and fibrinogen were dissolved in a suitable solvent in different concentrations and final suspension was prepared by addition of hydroxyapatite. After preparation of the suspensions, the conditions of the systems, which were film casting, and electrospinning, were set and scaffolds were produced.

The second part of the study includes the microstructure, phase and molecular bond characterization. It was conducted by scanning electron microscope (SEM), X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR), respectively, for characterization of the scaffolds.

2.1 Materials and Laboratory Equipment

2.1.1 Equipment

The laboratory equipment used during this study is listed in Appendix A,

2.1.2 Chemicals, Proteins and Buffers

The chemicals, proteins and chitosan are given in Appendix B together with their suppliers. The compositions and preparation of buffers and solutions are given in Appendix C.

2.2 Methods

2.2.1 The general outline of the experimental procedure:

- Preparation and characterization of hydroxyapatite by calcination
- Preparation of CH-HA-Fib films by film casting
- Preparation of CH-HA-Fib porous scaffolds by lyophilization and electrospinning
- Characterization of the specimens by XRD, FTIR and SEM

2.2.2 Preparation of hydroxyapatite by calcination and characterization:

Biologically derived hydroxyapatite powder was prepared by calcination of bovine bone at 850°C for 4 hours. The powder was grinded for 24 h in ethanol by ball milling and sieved to get homogenous particle size distribution.

Determination of the powder particle size and surface area was carried out using Mastersizer-Hydro 2000G and BET (NOVA 2200E), respectively.

2.2.3 Preparation of the scaffolds:

2.2.3.1 Preparation of CH-HA-Fib film

The suspensions for the film production were prepared in different concentrations. For this purpose, chitosan was dissolved in 2% (v/v) AcOH and hydroxyapatite suspension was prepared in distilled water at 2000 rpm on a mechanical stirrer for 1 hour. After, these two suspensions were brought together and new mix was stirred at 2000 rpm for 4 hours.

For specimens that contain fibrinogen, fibrinogen was dissolved in 1X PBS and added to the new suspension, and kept on the stirrer for one more hour. Moreover, pH of the final product was adjusted to 7.4 by NaOH.

The suspensions were casted into Petri dishes and dried for 4 days at room temperature.

All procedure was applied for five different compositions, which are given in Table 2.1.

Table 2.1: Different CH-HA and CH-HA-Fib films compositions.

NO	Chitosan (%w/v)	Hydroxyapatite (%w/v)	Fibrinogen (%w/v)
1	70	30	-
2	70	30	0.02
3	60	40	-
4	60	40	0.02
5	50	50	-
6	50	50	0.02
7	40	60	-
8	40	60	0.02
9	30	70	-
10	30	70	0.02

2.2.3.2 Preparation of CH-HA-Fib scaffold by lyophilization

The suspensions for the film production were prepared in different concentrations. For this purpose, chitosan was dissolved in 2% (v/v) AcOH and hydroxyapatite suspension was prepared in distilled water at 2000 rpm on mechanical stirrer for 1 hour. After, these two suspensions were brought together, the new mix was stirred at 2000 rpm for 4 hours.

The specimens that contain fibrinogen, fibrinogen was dissolved in 1X PBS and added to the new suspension and kept on the stirrer for one more hour. Moreover, pH of the final product was adjusted to 7.4 by NaOH.

All specimens, compositions of which are given in Table 2.2, were frozen at -20°C for 24 hours and lyophilized using a lyophilizator (Alpha 1-2 LD plus), shown in Figure 2.1, at -40°C under 0.012 mbar pressure for 6 days.

Table 2.2: Different CH-HA and CH-HA-Fib scaffolds compositions.

NO	Chitosan (%w/v)	Hydroxyapatite (%w/v)	Fibrinogen (%w/v)
1	70	30	-
2	70	30	0.02
3	60	40	-
4	60	40	0.02
5	50	50	-
6	50	50	0.02
7	40	60	-
8	40	60	0.02
9	30	70	-
10	30	70	0.02



Figure 2.1: Lyophilizator, Alpha 1-2 LD Plus.

2.2.3.3 Preparation of CH-HA-Fib scaffold by electrospinning:

Production of CH-HA-Fib nanofibers by electrospinning, Trifluoroacetic acid (TFA), 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP) and minimal essential medium (MEM) were used as solvents.

The specimens without fibrinogen contained chitosan and hydroxyapatite, firstly, chitosan was dissolved in neat TFA in different concentrations (6%, 8%, 10% (w/v)). Nanofiber formation was observed with SEM and the best result (10%) was chosen for future experiments. After choosing the best concentration, hydroxyapatite was added to the solution with different amounts (1%, 2%, 3% (w/v)) as listed in Appendix C.

The solution was pumped at the rate of 0,4 ml/h and high-voltage of 17-18 kV with the aluminum foil as a collector placed 10 cm away from the tip of the needle for electrospinning process.

Chitosan-Fibrinogen solutions were prepared in different concentrations. Beside the concentrations, the content of solvent included HFIP, TFA and MEM in different ratios as listed in Appendix C.

The solutions were pumped with different rates between 0,2-1 ml/h, different voltage values between 15-20 kV with the aluminum foil placed 10 cm away from the tip of the needle for electrospinning process.

2.2.4 Characterization of scaffolds

2.2.4.1 Phase analysis

The phase analysis of the products was conducted with X-ray diffractometer (Rigaku Miniflex) and thin film X-ray diffraction (Philips PW 1830) between 2θ : 20-80° with 2°/min speed and Cu-K α radiation.

2.2.4.2 Molecular bond characterization analysis

The molecular bond characterization of the products was conducted with Fourier transform infrared (Perkin Elmer Spectrum One) spectrum and the peaks were matched with literature.

2.2.4.3 Microstructure characterization

The microstructures of the scaffolds were observed by field emission scanning electron microscope (JEOL JSM 7000F) which is shown in the Figure 2.2.



Figure 2.2: Field emission scanning electron microscope, JEOL JSM 7000F.

2.2.4.4 Swelling test

Determination of the swelling ratio of the samples; each sample were balanced, when they were dry and incubated in 0,1 M PBS for one hour. In order to calculate the swelling ration the samples were balanced at 5', 10', 15', 30', 45' and 60' and the formula (2.1) below was used.

$$\frac{Ws-Wd}{Ws} \times 100 \quad (2.1)$$

where Ws is weight of wet sample and Wd is weight of dry sample.

3. RESULTS AND DISCUSSION

3.1 Characterization of the hydroxyapatite powder:

The HA powder was prepared by calcination method at 850°C for four hours and grinded for 24 h in ethanol by ball milling and sieved to get homogenous particle size distribution. The particle size distribution is given in Figure 3.1 and the average of particle size is 1.378 μm with 6,59 m^2/g surface area, which will be discussed in microstructure characterization section due to the effect of particle size of hydroxyapatite on nanofibers.

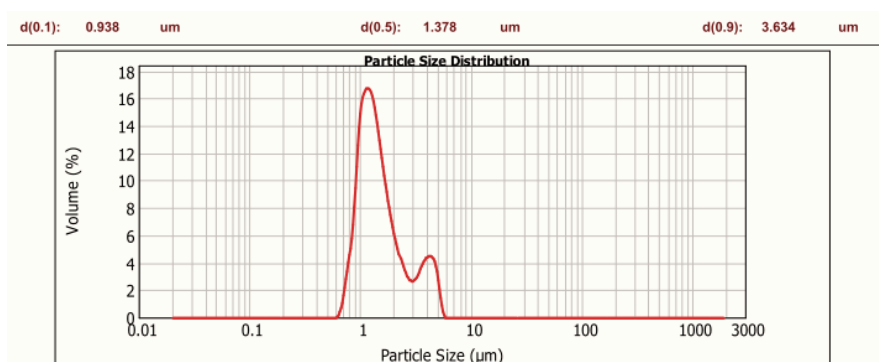


Figure 3.1: Particle size distribution of HA powder.

The phase analysis of the powder was performed with XRD and the peaks of the powder were matched with hydroxyapatite peaks at $2\theta=32.14^\circ$ and Peaks of the XRD spectrum reported in JCPDS 9–0432 file, which corresponds to hydroxyapatite. On the other hand, there are no peaks, which correspond to β -TCP. Therefore, the material produced was biologically derived hydroxyapatite powder (Figure 3.2).

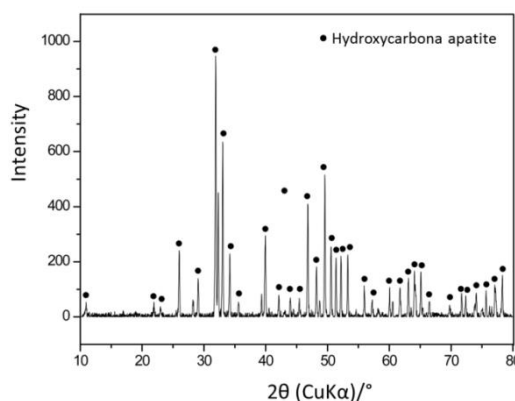


Figure 3.2: XRD pattern of pure HA powder.

3.2 Characterization of Chitosan-Hydroxyapatite-Fibrinogen Scaffolds:

3.2.1 Phase analysis:

All matters consist of atoms, in some cases these atoms arrange into a regular repeatable lattice, which is named crystal structure [20]. The crystal structure of a material is determined by X-Ray diffraction. In respect of XRD results, the atomic arrangement of chitosan, which is amorphous, is shown in Figure 3.3.

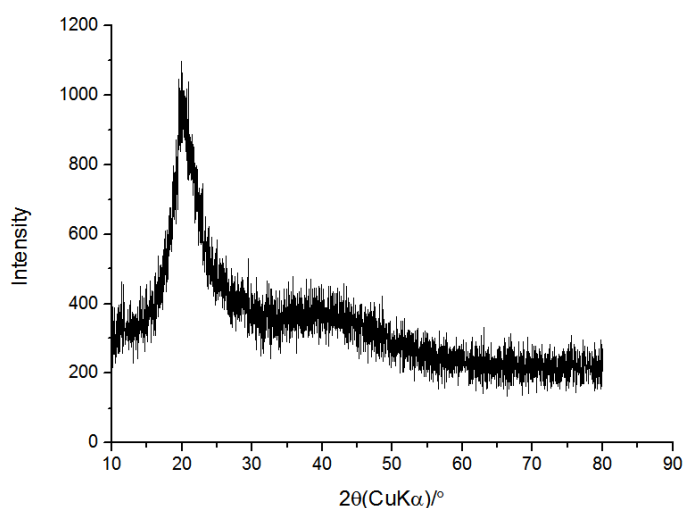


Figure 3.3: XRD pattern of pure low molecular weight chitosan.

According to the XRD patterns (Figure 3.4), the hydroxyapatite peaks decrease due to the presence of chitosan and by increasing the concentration of chitosan, the intensity of the peaks decreases in films. In Figure 3.4(a) and Figure 3.4(b), the concentrations of chitosan are high, thus the amorphous structure is more distinctive. However, all patterns show hydroxyapatite presence. All peaks are match with the 9-0432 card numbered hydroxyapatite peaks, which is hydroxycarbona apatite. These peaks show that the acidic environment and presence of the other compounds did not affect the crystal structure of the hydroxyapatite.

The XRD patterns of specimens, which were produced by lyophilization, are given in Figure 3.5. In order to determine the crystal structure of lyophilized scaffolds, thin film XRD was used. As shown in Figure 3.5 all specimen

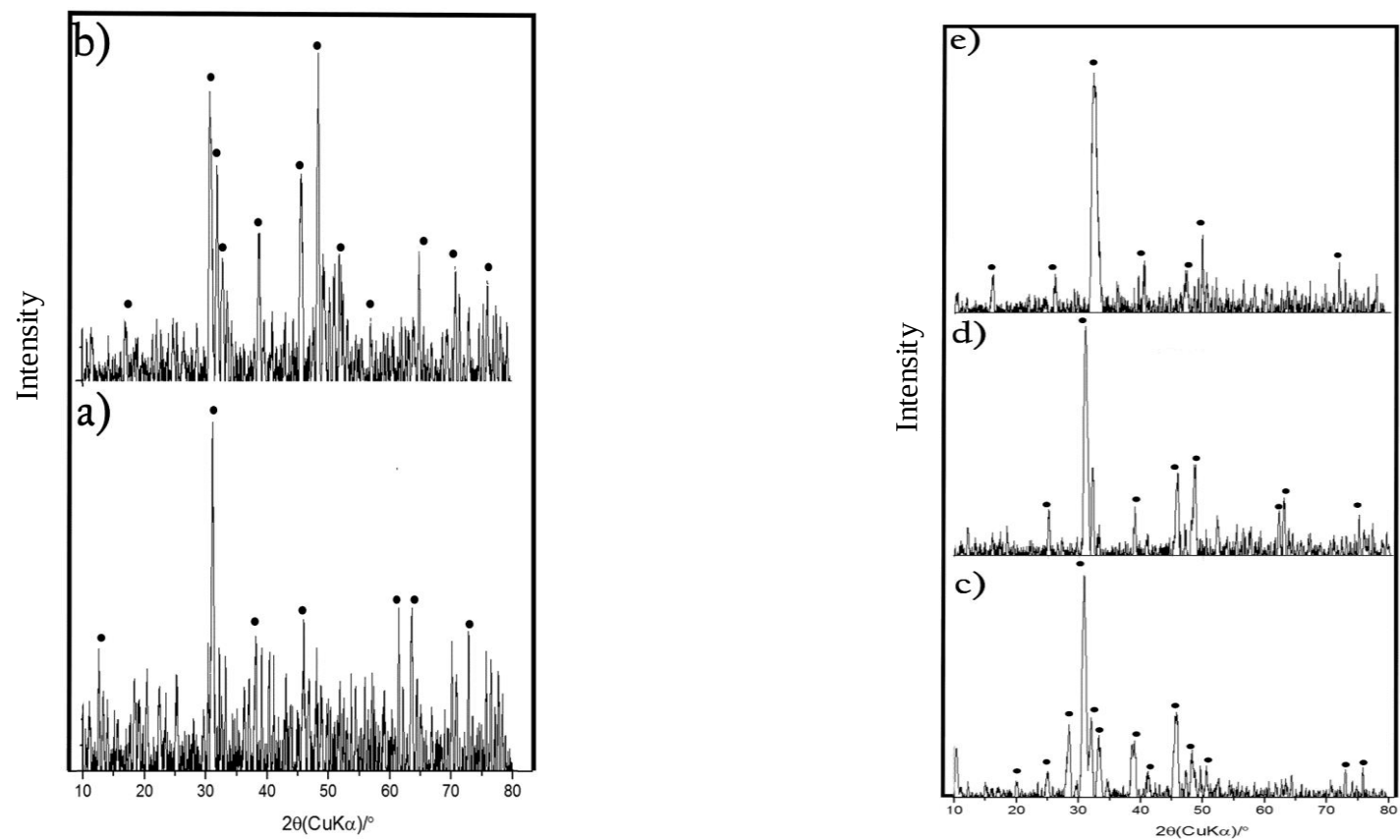


Figure 3.4: XRD patterns of a)70CH30HA, b)60CH40HA, c)50CH50HA, d)40CH60HA, e)30CH70HA films

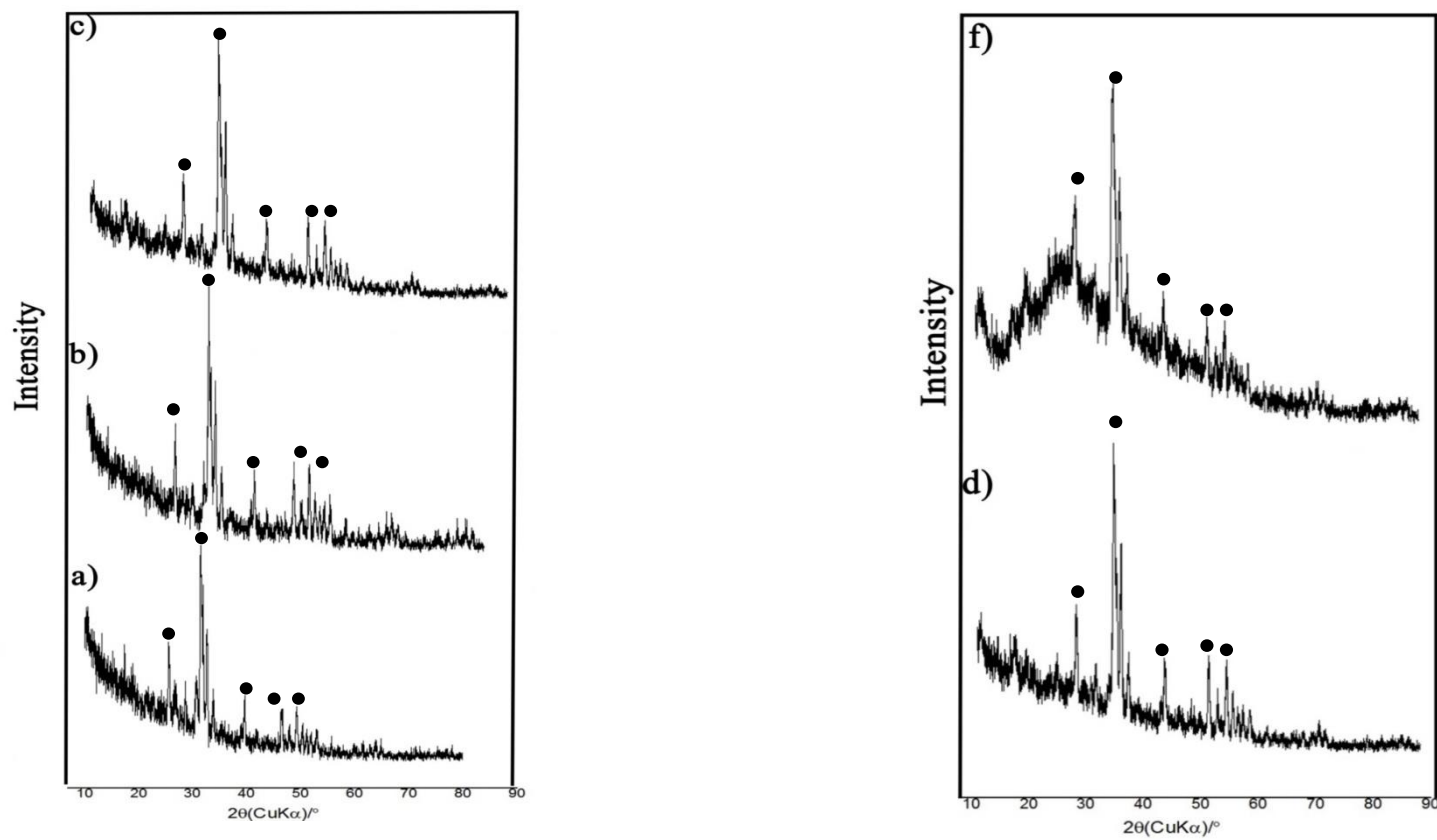


Figure 3.5: XRD patterns of a) 30CH70HA b) 40CH60HA c) 50CH50HA d) 60CH40HA e) 70CH30HA lyophilized scaffolds.

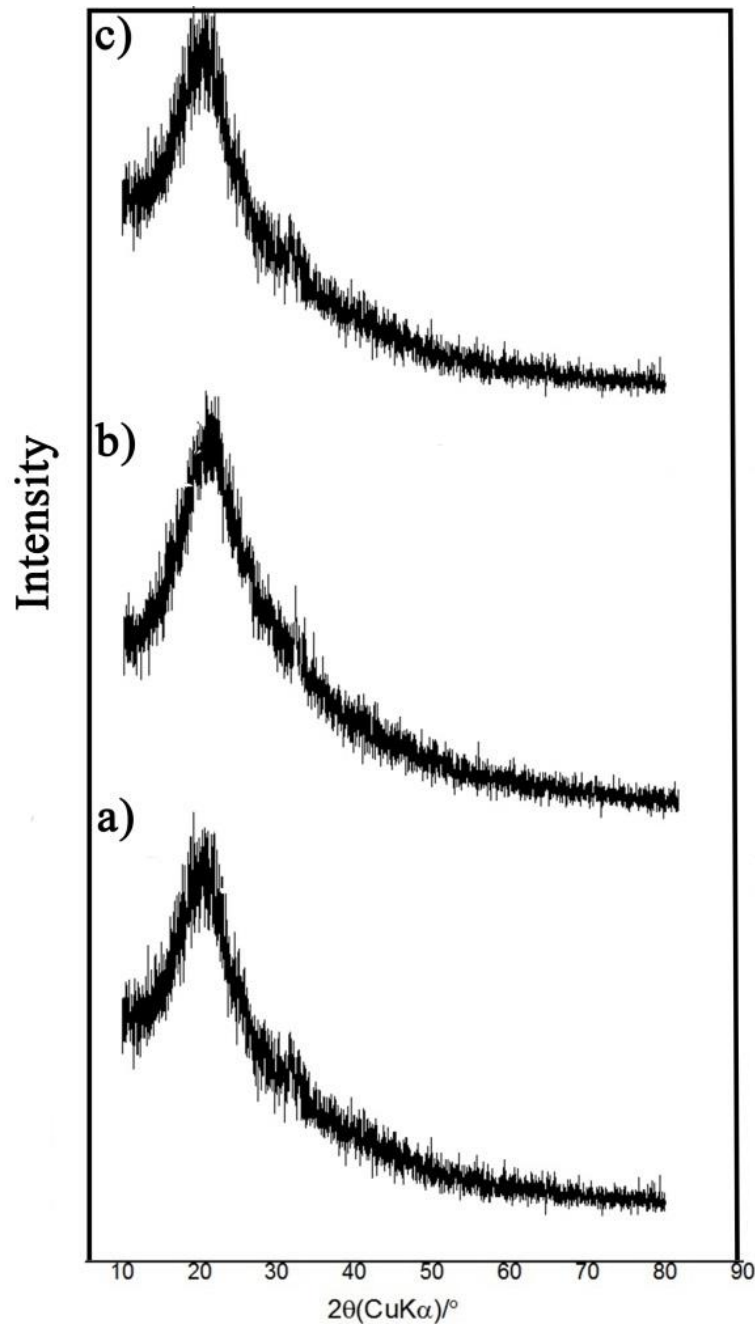


Figure 3.6: XRD patterns of a) 10CH1HA b) 10CH2HA c) 10CH3HA electrospinning scaffolds.

On the other hand in the XRD patterns of the scaffolds, which were produced by electrospinning, there is no HA peaks; although, the presence of hydroxyapatite was proved by molecular bond characterization, which will be explained next section (Figure 3.6). The reason of the amorphous structure could be the using of the neat TFA. This acid could be damage the hydroxyapatite structure. Either, the crystal structure in the scaffold could not be determined due to the low hydroxyapatite or high chitosan concentration or nanofibrous structure.

3.2.2 Molecular bond characterization

The molecular bond characterization of the specimens was performed by FTIR. Molecular bonds of chitosan, fibrinogen and hydroxyapatite, which are the beginning materials, are shown in Figure 3.7.

Figure 3.7(a) shows pure hydroxyapatite, which has PO_4^{3-} specific peaks around 1027 cm^{-1} [21].

Figure 3.7(b) is for pure fibrinogen molecular bond characterization. The dominant peaks around 1650 cm^{-1} defines amide I and 1550 cm^{-1} defines amide II, which show secondary structure of the protein and include C=O, N-H and C-N stretching vibrations, which belong to the amide groups. The third peak at 1327 cm^{-1} , amide III, shows the side chains of the amino acids and assigned to CH_2 vibrations [22].

The peaks at 3500 and 3000 cm^{-1} can be attributed to the $-\text{OH}$ and $-\text{CH}_3$ groups in Figure 3.7(c). The bands around $1600\text{-}1500\text{ cm}^{-1}$ are for amide I and amide II groups, which include N-H, C-H, C=O interactions and the peak at 1010 cm^{-1} defines C-O-N and C-O groups [23].

The film products that consist of hydroxyapatite and chitosan in different concentrations are shown in Figure 3.8. All concentrations have the same spectrum pattern, thus all of them can be discussed together. According to FTIR spectrum, besides N-H, C-H interactions and O-H stretching and bending, P-OH functional group appeared after CH-HA film production [24].

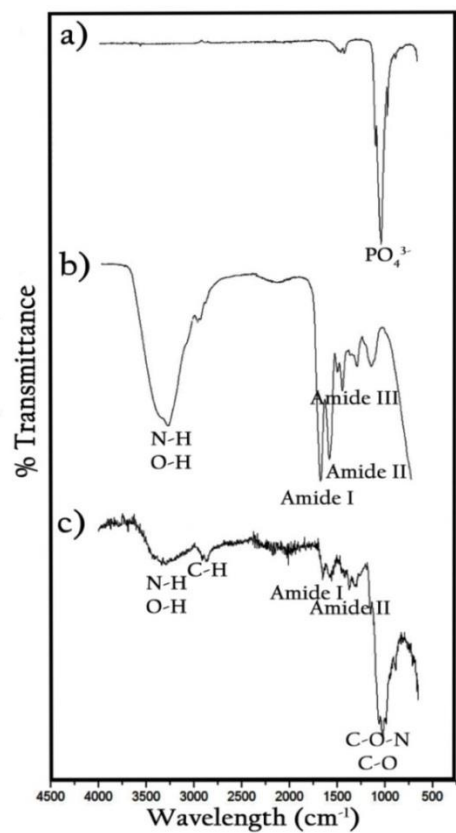


Figure 3.7: FTIR spectrum of a) pure HA, b) pure Fib., c) pure CH.

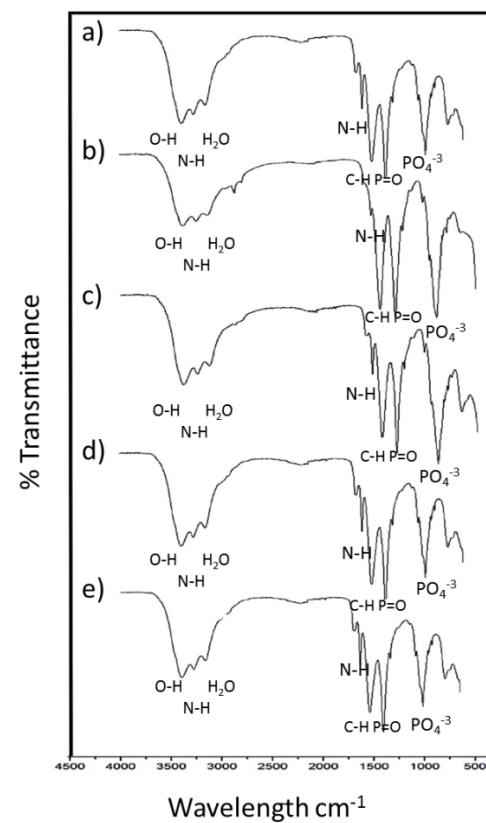


Figure 3.8: FTIR spectra of a) 30CH70HA, b) 40CH60HA, c) 50CH50HA, d) 60CH40HA, e) 70CH30HA films.

The FTIR spectrums of films containing fibrinogen (CH-HA-Fib) are shown in Figure 3.9. The amide I and amide II groups are present in all samples. On the other hand, the peaks around 3000-3500 cm^{-1} , correspond to water molecules and N-H, reduced by fibrinogen addition [23].

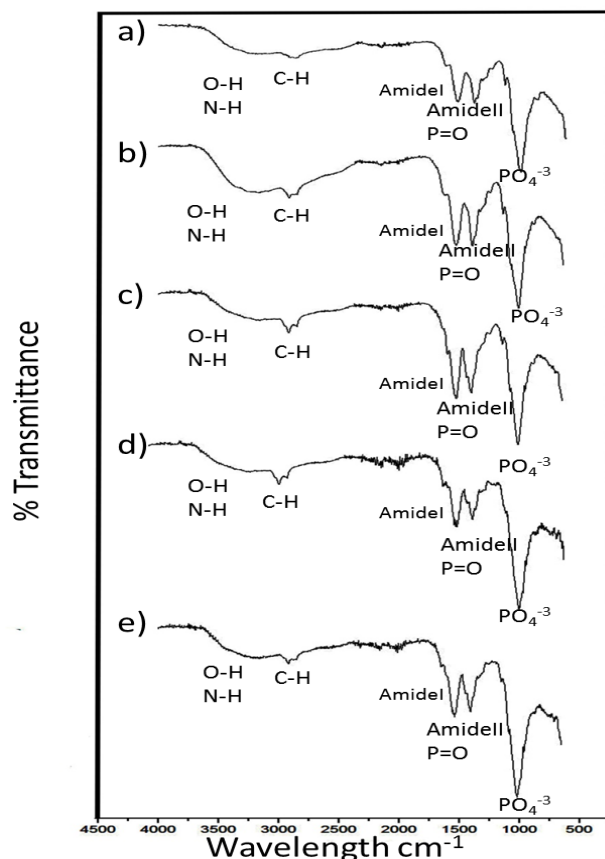


Figure 3.9: FTIR spectrums of a) 30CH70HAFib, b) 40CH60HAFib, c) 50CH50HAFib, d) 60CH40HAFib, e) 70CH30HAFib films.

Figure 3.10 and Figure 3.11 show the specimens that have been prepared with and without fibrinogen, respectively. In Figure 3.10 and Figure 3.11 all specimens have the same bonds, thus all bonds were evaluated together.

The lyophilized products, including fibrinogen, have the same bond with films except $-\text{OH}$ groups, thus whole water was removed from the product during the process [7]. Extra C-H, N-H and P-O interactions can be seen at 1500 and 3500 cm^{-1} in Figure 3.10.

The products without fibrinogen have almost same bands with same functional groups (Figure 3.11). The band at 1010 cm^{-1} shows the interactions between polymer and hydroxyapatite. The amide I and amide II groups of the chitosan and hydroxyl groups of hydroxyapatite overlap in both cases [25].

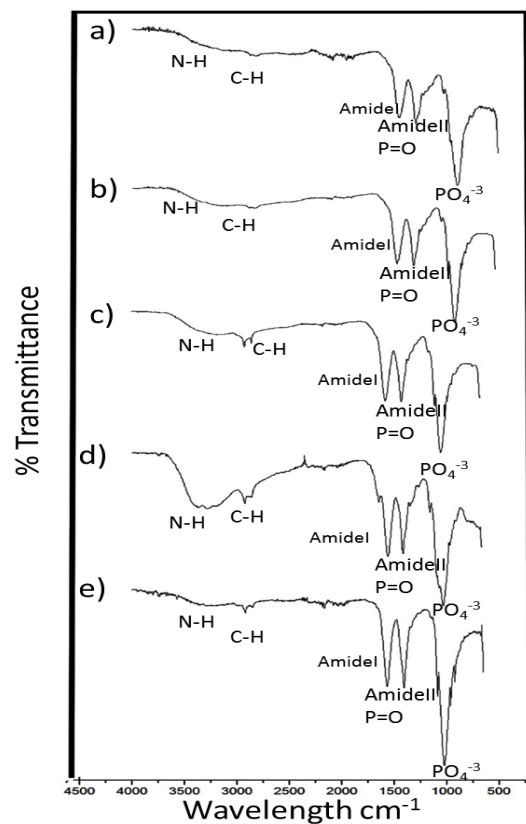


Figure 3.10: FTIR spectrums of lyophilized a) 30CH70HA, b) 40CH60HA, c) 50CH50HA, d) 60CH40HA, e) 70CH30HA scaffolds.

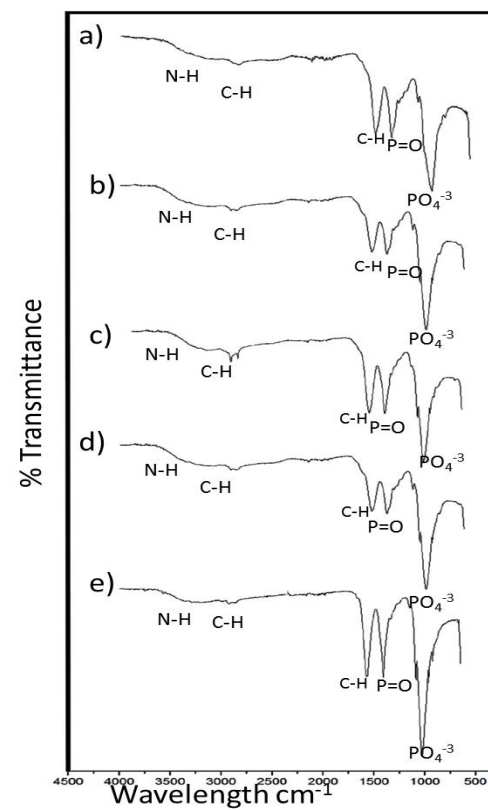


Figure 3.11: FTIR spectrums of lyophilized a) 30CH70HAFib, b) 40CH60HAFib, c) 50CH50HAFib, d) 60CH40HAFib, e) 70CH30HAFib scaffolds.

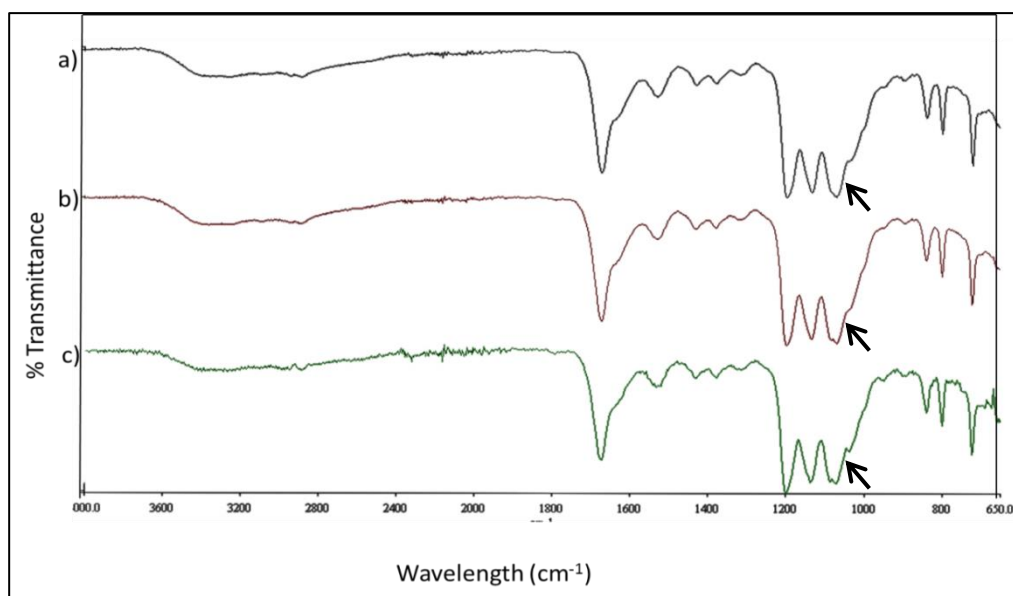


Figure 3.12: FTIR spectrums of electrospinning a) 10CH1HA, b) 10CH2HA, c) 10CH3HA scaffolds.

The hydroxyapatite specific bands, present between 900 and 1100 cm^{-1} , are shown in Figure 3.12. The bands, which are denoted with arrows ($950\text{--}1085\text{ cm}^{-1}$), show the interactions between chitosan and hydroxyapatite according to Frohbergh et.al (2012) [26].

3.2.3 Microstructure characterization:

The CH-HA-Fib scaffolds were prepared by three different techniques and microstructural differences microstructures of the scaffolds were determined by SEM. The character of a scaffold is determined by its porosity, pore size, pore shape and interconnectivity since these parameters could affect the cell viability, both *in vivo* and *in vitro* [27]. The small pore size prevents cell migration into the scaffold in contrast to large pore size, which prevents cell adhesion to the scaffold [28], thus there should be an optimum pore size, which is $>150\text{ }\mu\text{m}$ for osteoblast ingrowth [27].

Three types of chitosan-hydroxyapatite and fibrinogen based scaffolds were prepared and their microstructure and porous structure were determined by SEM that are shown in Figure 3.13, Figure 3.14 and Figure 3.15 as film, sponge and nanofibers, respectively.

As it is shown in Figure 3.13, there is a clear difference between the specimens with and without fibrinogen. The productions without fibrinogen (a, b, c, d, and e) have

pore-like structure but the size and shapes of these holes may not be suitable for cell adhesion and migration as mentioned before. Since, the pore size is too small for cells to migrate inside the film. On the other hand, the SEM images just the surface images and it is difficult to observe the inside of the film, so it could not be known that if these pore-like structures go into the film and have connection between each other. If they do not, beside the cell migration, the cell culture transportation will be difficult.

The productions with fibrinogen (Figure 3.13f, g, h, i and j) have a bulk structure that is caused by N-H, C-H interactions between chitosan and fibrinogen, which will be explained in the molecular bond characteristic analysis section. It is obvious that there is no pore formation in these specimens and fibrinogen spread to all film, which may obstruct the interaction between fibrinogen and the cells.

The microstructure of productions, which were produced by technique at -40°C under 0.012 mbar pressure for 6 days, are shown in Figure 3.14.

According to the lyophilization method, the polymer solution is frozen first and then water molecules are removed from the solution by sublimation under low pressure and low temperature conditions [15]. Porous structure, which provides a suitable environment for cell migration and adhesion, was obtained by lyophilization technique. The pore size was enlarged by decreasing the CH ratio in the scaffold until 50CH50HA, but after that ratio, the shape and size of pores may have been deformed due to the high concentration of hydroxyapatite.

The pore morphology in Figure 3.14 (a, b, f and g) is caused by hydroxyapatite particles were extruded among the ice crystals [29]. However, the SEM images indicate that porous structure of the specimens may obtain the environment, which is required for adhesion and migration of the cells and blood circulation.

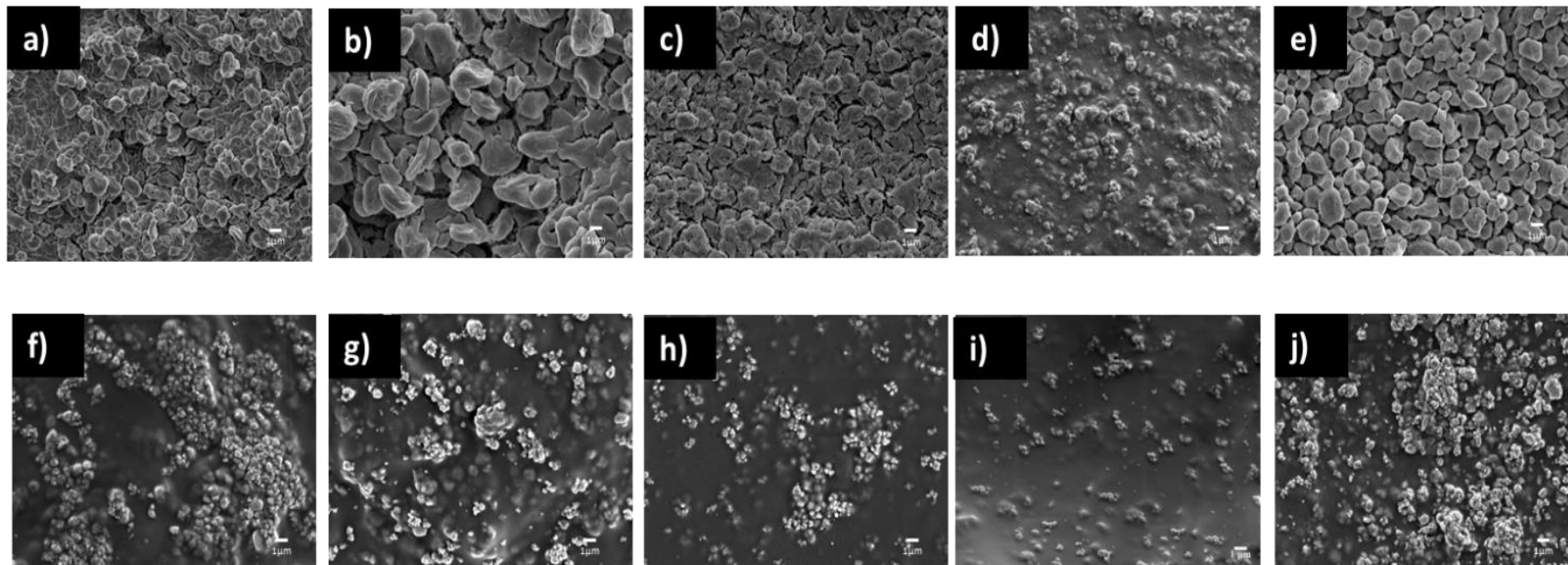


Figure 3.13: The SEM images of a) 30CH70HA, b) 40CH60HA, c) 50CH50HA, d) 60CH40HA, e) 70CH30HA, f) 30CH70HAFib, g) 40CH60HAFib, h) 50CH50HAFib, i) 60CH40HAFib, j) 70CH30HAFib films.

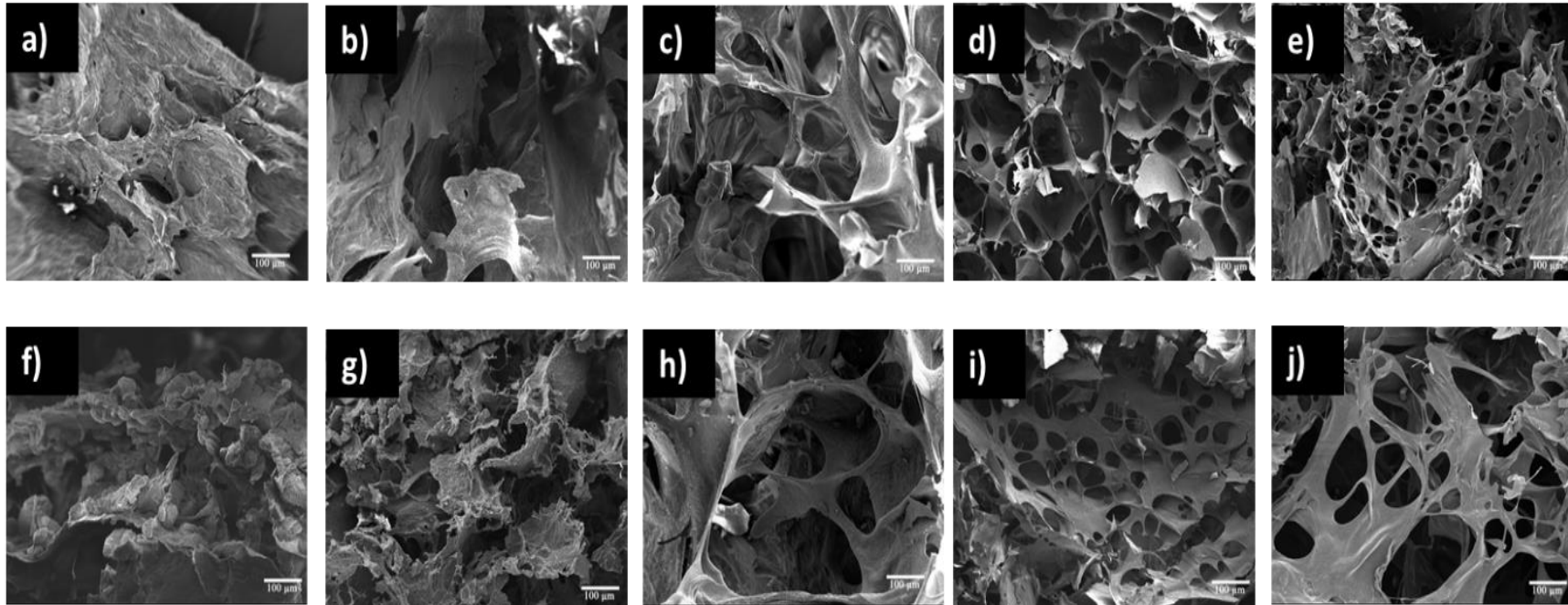


Figure 3.14: The SEM images of a) 30CH70HA, b) 40CH60HA, c) 50CH50HA, d) 60CH40HA, e) 70CH30HA, f) 30CH70HAFib, g) 40CH60HAFib, h) 50CH50HAFib, i) 60CH40HAFib, j) 70CH30HAFib lyophilized scaffolds.

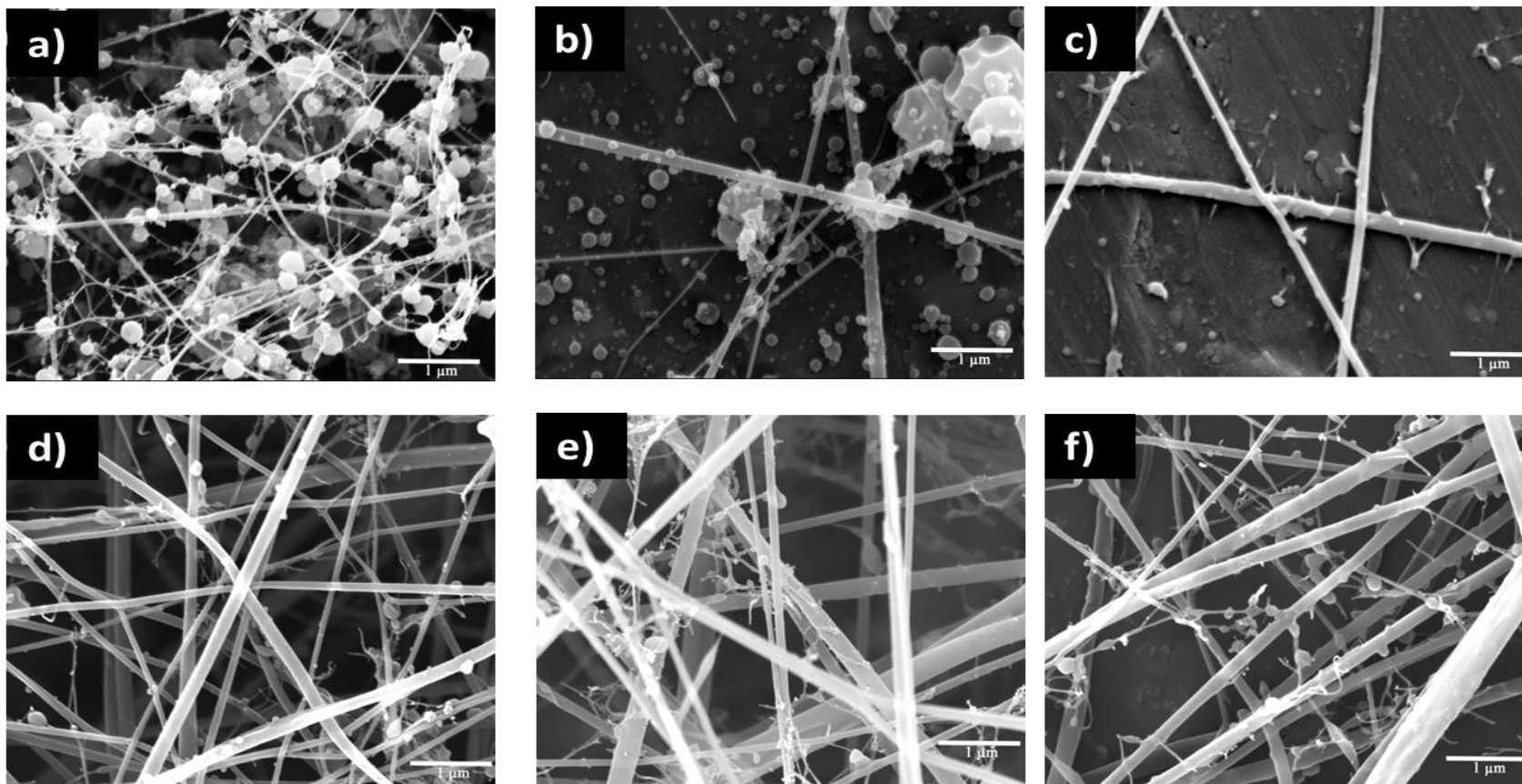


Figure 3.15: The SEM images of nanofibers a) 6% CH in TFA, b) 8% CH in TFA, c) 10% CH in TFA, d) 10% CH and 1% HA in TFA, e) 10% CH and 2% HA in TFA, f) 10% CH and 3% HA in TFA.

To produce the chitosan based nanofibers TFA is used as the solvent [30]. Different concentrations of the CH were chosen and the electrospinning conditions were changed to produce finest nanofibers. Firstly, 6%, 8% and 10% of chitosan concentrations were applied electrospinning process. As shown in Figure 3.15(a) 6%CH nanofibers are in bead-fiber structure. The bead formation lead to the conclusion that the concentration of the solutions should be increased [30]. Although the bead formation decreased by increasing the concentration of the solution to 8%, fine nanofibers were performed with 10% of chitosan under 0,4 ml/h pumping rate, 17 kV and 10 cm distance between needle and collector as the conditions. The fabrication of nanofibers instead of beads is very important due to the architectural similarity with extracellular matrix, which gives cells opportunity of migration and adhesion. Besides the fluid of the body and blood can transport into the scaffold and provides nutrient environment to the cells [32].

After the production of nanofibers, chitosan-hydroxyapatite compositions were prepared as listed in Appendix C and the same electrospinning conditions were applied. The nanofibers were produced successfully as shown in Figure 3.15(d, e, f). According to Mi et al. while nano HA powder increases the diameter of nanofibers, micro sized HA powder causes a decrease [21]. The specimen with 10% CH (w/v) and 1% HA (w/v) is shown in Figure 3.15(d), 2% HA (w/v) in Figure 3.15(e) and 3% HA in Figure 3.15(f). All images show that continuous nanofibers were produced after electrospinning process; however, there are very thin and short nanofibers, which may have been observed due to the micro sized hydroxyapatite.

Nanofibers were not formed from solutions with fibrinogen.

3.2.4 Swelling tests

Chitosan, a polysaccharide, is an insoluble natural polymer. Both crosslinked and uncrosslinked forms of the chitosan polymer can absorb water. The water molecules can be bound to the polymer tightly or present as free water [33]. Swelling is the easiest way to determine the crosslinks between molecules. During the swelling process, the linkages between molecules elongates because of the solvent absorbed by the polymer. This elongation continues until the forces on the linkages are balanced, which are elongation force caused by swelling and elastic retractive force obstructs elongation [33]. The swelling ratios of the CH-HA and CH-HA-Fib scaffolds are given in Figure 3.16 and Figure 3.17.

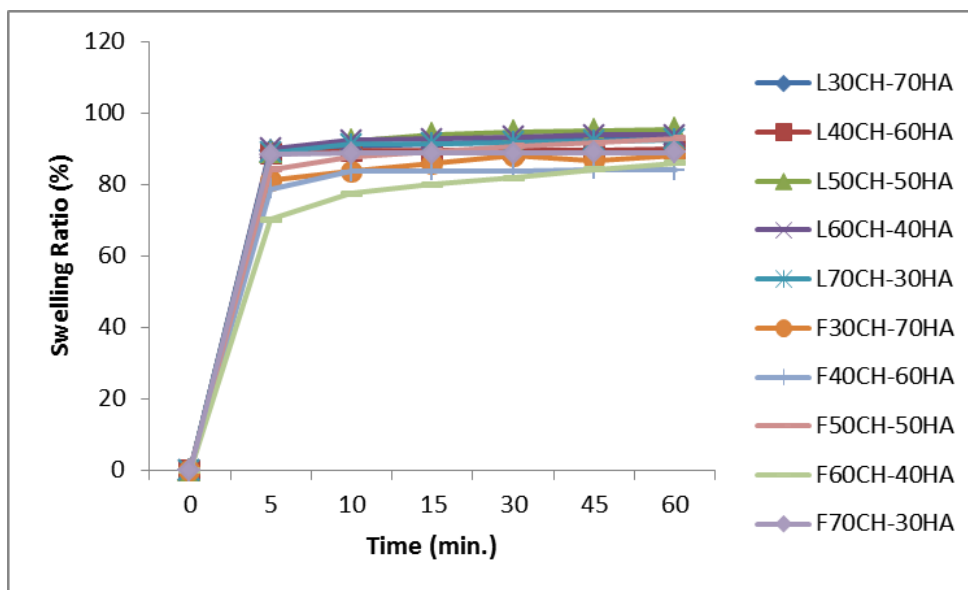


Figure 3.16: Swelling ratios of films and lyophilized scaffolds without fibrinogen.

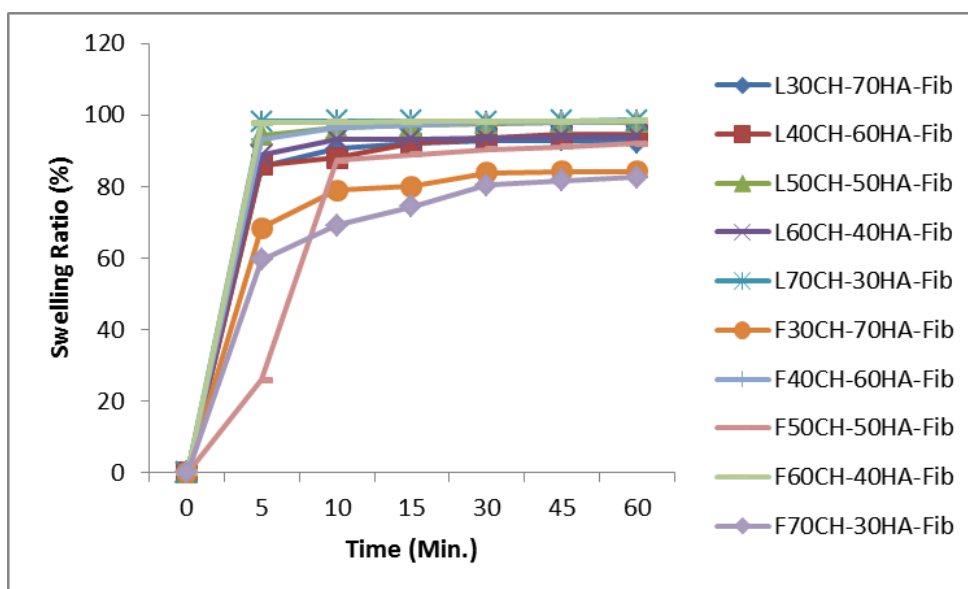


Figure 3.17: Swelling ratios of films and lyophilized scaffolds with fibrinogen.

According to the figures, given above, chitosan films and lyophilized scaffolds can swell in 0,1M PBS. On the other hand, the chitosan concentration and production method is not significantly effective on polymer swelling. Besides, adhesion of fibrinogen, which is a soluble protein, increases the swelling rate of polymers. The swelling ration can give information about molecular interactions and probable behavior of the scaffold in the organism. The PBS is chosen as buffer solution to determine the swelling ratio because of the salt concentration and pH, which are very close to physiological conditions. In this case, it is obvious that films and scaffolds can swell almost 100% in 60 minutes.

As mentioned in section 3.2.3, the molecular interactions between chitosan, fibrinogen and hydroxyapatite were determined by FTIR. These interactions affect the swelling properties of the polymer directly. Addition of fibrinogen may strengthen the interactions or cause the formation of new linkages. Therefore, the swelling ratio of the polymers may have increased with fibrinogen. On the other hand, the crosslinkages inside the fibrinogen may have been the reason of the increased swelling ratio.

4. CONCLUSION

During the studies, CH-HA and CH-HA-Fib films and scaffolds were produced in different concentrations by film casting, lyophilization and electrospinning methods. After production, phase analysis, molecular bond characterization and microstructure characterization were conducted by using of XRD, FTIR and SEM respectively and swelling tests were run in PBS for 60 minutes.

1. CH-HA and CH-HA-Fib films and lyophilized scaffolds; CH-HA electrospinning scaffolds were produced successfully.
2. The crystal structure of hydroxyapatite is preserved in films and lyophilized scaffolds but not in electrospinning scaffolds. The intensity of hydroxycarbona apatit peaks decreased with increasing chitosan composition.
3. The molecular interactions between chitosan, fibrinogen and hydroxyapatite were determined by FTIR and certain bands were observed at specific positions, which belong to C-H, N-H and P-O interactions.
4. The microstructures of films and scaffolds were observed by SEM. The lyophilized scaffolds have porous and electrospinning scaffolds have nanofibrous structure as expected. However, the porous structure of the lyophilized scaffolds degenerated by increasing hydroxyapatite concentration. This architecture of scaffolds obtains an ECM like environment for cells and increases the cell proliferation.
5. Although the production technique did not affect the swelling ratio, addition of fibrinogen increases the swelling ratio. All specimens with fibrinogen swelled almost 100% and without fibrinogen swelled almost 90%.

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APPENDICES

Appendix A

Appendix B

Appendix C

APPENDIX A

Laboratory Equipment

Balance	Precisa XB 220A
BET	NOVA 2200E
Electrospinning	Inovenso
FTIR	Perkin Elmer Spectrum One
Lyophilizator	Alpha 1-2 LD plus
Magnetic stirrer	Arex
Mechanic stirrer	Heidoph RZR 2020
SEM	JEOL JSM 7000F
Syringe	Set inject
Thin film XRD	Philips PW 1830
Pippette	Eppendorf 200 µl
pH Meter	WTW pH 330i
XRD	Rigaku Miniflex

APPENDIX B

Chemicals

1,1,1,3,3,3 Hexafluoro-2-propanol	Alfa Aesar
Acetic acid	Alfa Aesar
Ethanol	Emsure
Minimal essential medium (1M)	Gibco
NaCl	Merck
NaOH	Merck
Phosphate buffer saline (1M)	Gibco
Trifluoroacetic acid	Merck

Chitosan and Proteins

Chitosan	Sigma-Aldrich, Low molecular weight
Fibrinogen	Sigma-Aldrich, Pig Plasma

APPENDIX C

Buffers and Solutions

Acetic acid solution (2% v/v) (100ml)

- 2 ml Acetic acid
- 98 ml Distill Water

HFIP:TFA solution (90:10) (10 ml)

- 9 ml HFIP
- 1 ml TFA

HFIP:TFA solution (80:20) (10 ml)

- 8 ml HFIP
- 1 ml TFA

HFIP:MEM:TFA solution (85:10:5) (10 ml)

- 8,5 ml HFIP
- 1 ml MEM
- 0,5 ml TFA

NaOH solution (5% w/v) (30 ml)

- 1,5 g NaOH
- 30 ml Distill Water

PBS (0,1 M) (100 ml) (pH: 7,4)

- 10 ml 10X PBS
- 90 ml Distill Water

Table C.0.1: Polymer solutions for film casting and lyophilization methods.

Name	Chitosan (g)	Hydroxyapatite (g)	Fibrinogen (g)	AcOH (ml)
70CH30HA	1.4	0.6	-	50
70CH30HAFib	1.4	0.6	0.01	50
60CH40HA	1.2	0.8	-	50
60CH40HAFib	1.2	0.8	0.01	50
50CH50HA	1	1	-	50
50CH50HAFib	1	1	0,01	50
40CH60HA	0.8	1.2	-	50
40CH60HAFib	0.8	1.2	0.01	50
30CH70HA	0.6	1.4	-	50
30CH70HAFib	0.6	1.4	0.01	50

Table C.0.2: Polymer solutions for electrospinning method.

Name	Chitosan (g)	Hydroxyapatite (g)	Fibrinogen (g)	NaCl (g)	TFA (ml)	HFIP (ml)	MEM (ml)
6CH	1.2	-	-	-	20	-	-
8CH	1.6	-	-	-	20	-	-
10CH	2	-	-	-	20	-	-
10CH1HA	2	0.2	-	-	20	-	-
10CH2HA	2	0.4	-	-	20	-	-
10CH3HA	2	0.6	-	-	20	-	-
6CHFib	1.08	-	0.12	-	2	18	-
8CHFib1	1.44	-	0.16	-	2	18	-
8CHFib2	1.28	-	0.32	-	2	18	-
10CHFib1	0.8	-	0.2	-	1	9	-
10CHFib2	0.9	-	0.1	-	0.5	8.5	1
10CHFib3	0.8	-	0.2	0.02	1	9	-

CURRICULUM VITAE



Education

- M.Sc. Degree in Materials Engineering 2011-2014
Istanbul Technical University, Turkey (GPA: 3.38 out of 4)
- B.Sc. Degree in Molecular Biology and Genetics 2006-2011
Istanbul Technical University, Turkey (GPA: 2.53 out of 4)
- High School Diploma with Science Concentration 2002-2006
Niğde Anatolian Teacher High School, Turkey (GPA: 4.85 out of 5)

Research and Work Experience

- Graduate thesis work at the Department of Materials Engineering, Istanbul Technical University, advised by Prof. Dr. Gültekin Goller (June 2013-current)
Graduate Thesis (in progress): *Production of 3D Chitosan-Hydroxyapatite-Fibrinogen Scaffolds by Three Different Techniques*
- Undergraduate thesis work at the Department of Molecular Biology and Genetics, Istanbul Technical University, advised by Assoc. Prof. Fatma Neşe Kök (Spring 2011)
Undergraduate Thesis: *Stability of Phosphatidylcholine-Phosphatidylethanolamine Liposomes*
- Part-time assistantship at Department of Molecular Biology and Genetics, Istanbul Technical University (Fall 2010 and Spring 2011)
- Research internship on biomaterials and regenerative medicine at Assoc. Prof. Dr. Ali Khademhosseini's Laboratory at Harvard-MIT Health, Sciences and Technology Institute (June-August, 2010)
- Research internship at Prof. Dr. Gamze Torun Köse's Tissue Engineering Group at Department of Genetics and Bioengineering, Yeditepe University (January, 2010)
- Internship at Genmer Genetic Diseases Diagnostic Center (June-July, 2008)

Presentations

- **Türkmen A. K.** (2013) *Production of 3D Chitosan-Hydroxyapatite-Fibrinogen Scaffolds by Three Different Techniques* Graduate thesis proposal (MAM596-Seminar course) delivered at the Department of Materials Engineering, Istanbul Technical University.
- **Türkmen A. K.** (2011) *Stability of Phosphatidylcholine-Phosphatidylethanolamine Liposomes.* Undergraduate thesis defense delivered at the Department of Molecular Biology and Genetics, Istanbul Technical University.

Language Skills, Honors, Distinctions and Scholarships

- Turkish: Native; English: Fluent
- Graduate student in the framework of the bi-national research project between Turkey and Romania: *A New Generation of Alumina/Zirconia Biocomposites for Dental and Orthopedic Implants: Production, Characterization and in vitro/in vivo Evaluation*
- Appeared on Istanbul Technical University's Honor List
- Istanbul Metropolitan Municipality University Scholarship (2007)

Attended Workshops

- 4. Stem Cell Symposium, Turkish Academy of Sciences, Ankara (June 26-27, 2009)
- HORIZONS in Molecular Biology and Genetics Symposium, Bilkent University, Ankara (May 11-12, 2009)
- Nanobiomat, Tissue Engineering and Nanobiotechnology Workshop, Middle East Technical University, Ankara (September 1-2, 2008)
- Molecular Biology and Genetic Spring Symposium, Science and Technology Student Organization, Haliç University, İstanbul (May 2-4, 2008)