LEILA KARIMIZARANDI SYNTHESIZE AND CHARACTERIZATION OF SILK FIBROIN/CHITOSAN/ EGGSHELL HYBRID SCAFFOLD AND BIOFILM NEU 2018

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A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF APPLIED SCIENCES OF NEAR EAST UNIVERSITY

BY
LEILA KARIMIZARANDI

In Partial Fulfillment of the Requirements For The Degree of Master of Science in Biomedical Engineering

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Approval of Director of Graduate School of Applied Sciences

Prof. Dr. Nadire Çavuş

We certify this thesis is satisfactory for the award of the degree of Masters of science in Biomedical Engineering

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I hereby declare that all the information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and refer and referenced all material and results that are not original to this work.

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Date:

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In the name of the most merciful and majesty who gave me all blessing…
I would love to thank my parents and my family for all the support, patient and kindness.
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And finally, last but not least to everyone who helped and supported me and it was great sharing knowledge and cooperating with all of you during this year.

To My Parents…

ABSTRACT

The main aim of this study is to synthesize and characterize silk fibroin /Chitosan/Eggshell blended scaffold and film by using direct drying method, hydrogel and sol-gel technique with Tripolyphosphate (TPP 5 %wv) solution as crosslinker by Ethanol:PBS 40%:60% v/v (PH:7.4) for surface treatment. Green waste is the best source in industrial and medical areas due to their low cost and great supply on earth. Combination of Chitosan (Cs) as a polysaccharide, Silk fibroin (S.F) as natural protein and poultry eggshell (ES) as mineral bioceramic make biosafe, biocompatible and biodegradable biomaterial to synthesize 3-dimension bulk as the scaffold and bioactive planar structure as film specifically in bone regeneration. Cs/SF/ES meshwork and biofilm have been tested by Peripheral Streaming method for platelet adhesion analysis, biodegradation in protease enzyme and swelling behaviors were analyzed. FTIR was performed.

Keywords: Silk Fibroin; Platelet Adhesion; Blood Compatibility; Chitosan; Tripolyphosphate

ÖZET

Yeşil atık, dünyadaki düşük maliyet ve büyük arz nedeniyle endüstriyel ve tıbbi alanlarda en iyi kaynaktır. Kitosan (Cs), İpek fibroin proteini ve yumurta kabuğu (ES) mineral biyoseramik olarak biyouyumluluğu yüksek, biyobozunur özellikleri ile biyofilim ve 3B skele olarak özellikle kemik regenerasyonunda kulanımı uygundur. Bu çalışmanın temel amacı, kitosan / ipek fibroin /ES hibrid sistemin 3B iskele ve biyofilm olarak sentezlenip kan uyumluluğu özelliğinin karakterize edilmesidir. Doğrudan kurutma yöntemi, sol/ gel tekniği kullanılarak tripolifosfat (TPP % 5 w/v) çözeltisi ile etanol: PBS (fosfat tampon çözeltisi; pH = 7.4) kullanılarak hazırlanmış biyofilm ve 3B iskeleler farklı konsantrasyonlarda hazırlanmışlardır. Periferik streaming yöntemi kullanılarak, biyofilm ve 3B iskelelere yapılan analizlerde, platelet adhezyonunun olmadığı gözlemlenmiştir.

Cs/İF/ES biyofilm ve 3B iskelelerinin kan uyumluluğunun yüksek olduğu sonucuna varılmıştır. Biyomateryallerin kan teması olan cihaz veya protezlerde kullanımı uygun olarak tespit edilmiştir. Fourier dönüşümlü infrared spektrofotometre uygulandı.

Anahtar Kelimeler: İpek Fibroin; Trombosit Adezyonu; Kan Uyumluluğu; Kitosan; Tripolifosfat

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LIST OF ABREVIATIONS

AA:	Acetic Acid
App:	Application
AW:	Apatite-Wollastonite
BCP:	Bathocuproine
BG:	Bioactive Glass
BI:	Bone Implantation
BMP:	Bone-Morphogenetic-Protein
C57B6:	C57 Black 6 - Laboratory Mouse
CAD:	Computer Aided Design
CaR:	Calcium Sensing Receptor
CP:	Calcium Phosphate
Cs:	Chitosan
CS:	Calcium Sulfate
DBM:	Demineralized Bone Matrix
DW:	Distilled Water
ECM:	Extracellular Matrix
EDTA:	Ethylene diamine tetra acetic Acid
ES:	Eggshell
ESM:	Eggshell Membrane
FACIT:	Fibril-Associated Collagens With Interrupted Triple Helices

FDA:	Food And Drug Administration
FGF:	Fibroblast Growth Factor
FTIR:	Fourier Transform Infrared Spectrophotometer
G.F:	Growth Factor
G:	Glycerin
G6PD:	Glucose-6-Phosphate-Dehydrogenas
GAGs:	Glycosaminoglycan
GMP:	Good Manufacturing Practice
HA:	Hydroxyapatite
HAS:	Human Serum Albumin
HFIP:	Hexa fluoro-isopropanol
IGF:	Insulin-Derived Growth Factor
MARK:	Mitogen-Activated Protein Kinase
MMA:	Methyl Methacrylate
MSC:	Mesenchymal Stem Cells
PBT:	Polybutylene Terephthalate
PCL:	Polycaprolactone
PDGF:	Platelet-Derived Growth Factor
PDLA:	Polyd-Lactic Acid
PDS:	Polydioxanone
PEG:	Polyethylene Glycol

PGA: Polyglycolide

PHE: Poly-A Hydroxyl-Ester

PHEMA: Polyhydroxyethylmethacrylate

PHPMA: N-(2-Hydroxypropyl) Methacrylamide

PLGA: Poly-DL-Lactic-Coglycolic Acid

PLLA: PolylacticAcid

PPF: Poly(Propylene Fumarate)

PVA: Polyvinyl Alcohol

QCT: Quantitative Computed Tomography

QMRI: Quantitative Magnetic Resonance Imaging

S.F: Silk Fibroin

SEVA-C: Starch Ethylene Vinyl Alcohol

SFF: Solid Free Form

T.E: Tissue Engineering

T: Temperature

TCP: Tricalcium Phosphate

TGF-*β*: Transforming Growth Factor-*B*

TPP: Tripolyphosphate

US: Ultrasound Scanning

CHAPTER 1

INTRODUCTION

1.1 Introduction

Utilizing natural waste, the best source of raw material for synthesizing crystalline, hydrogel, nanoparticle in different subjects of science, especially in biomedical sector, has become an important and vital factor in the present times. In/organic waste or green waste is considered as the most essential and low cost resource for innumerable purposes. Investigation on recovering and recycling these materials and reusing them in biodegradable, biocompatible and non-toxic biomaterial application, including scaffold and film (biofilm), has proved its efficiency in producing a variety of products in medical fields. Figure 1.1 is shown the outline of this research.

One of the most common agricultural waste is poultry eggshell (ES). ES contains bioceramic properties as bone tissue and is the most abundant, cheap and environmental friendly.

Biomedical application of biodegradable polysaccharide in areas such as gel, capsules, fiber, biofilm, sponge and bioresorbable scaffold has already proved as useful application. Different concentrations of biodegradable polysaccharide resulted in diverse forms of products. Chitosan (Poly-β-1-4-2-amino-2-deoxy-D-glucose) (Cs)- amino based, is taken from chitin. Cs is another green waste biomaterial that is studied in this research.

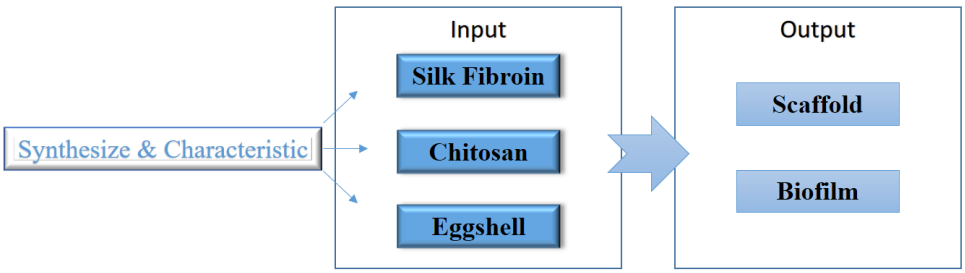


Figure 1.1: Outline

Commercial and natural protein resources that are obtained from silkworm is another ingredient in this study. Silk fibroin (S.F) has many applications. Its unique biological characteristics and structure make it one of the potential biomaterial in tissue engineering (T.E).

Bone tissue engineering aims to function through an emerging method using biomaterial factor therapy of cells. For achieving this purpose, it requires biocompatible scaffold to imitate the properties and structure of natural bone. (Amir Amini et al., 2013) Bone is the hardest tissue in the biological system. Functioning as structural support and vital protection, it is a storage of minerals and provide blood cells. Through time bone density decreases and the bone cannot renew itself. Bone diseases from a simple fracture to serious issues such as osteogenesis imperfect, osteoporosis, bone cancer etc. need to be treated by applying new methods using tissue engineering application. Although some biomaterials are not fulfilling all requirements and modification for such procedures, it should work on all criteria in the long term picture.

The aim of this research is based on synthesizing the characteristics of bioceramic scaffold and film based on agricultural waste resources for bone regeneration using direct dry method and hydrogel and sol-gel technique. Designing and synthesizing of composite biomaterial make the opportunity to get scaffold and film with mechanical, physical, chemical and biological possessions by addition polymers and bio-active materials; Table 1.1 is shown some bioactive materials which react into biological system-body to form HA as a layer that leads to shape of a firm tissue like bone with hard and soft tissues.

Table 1.1: Bioactive inorganic materials (Faezeh Hajiali et al., 2017)

Bioactive material	1.Ceramic	CaP/HA/TCP
	2.Glass	BG
	3.Glass-ceramic	AWand b-wollastonite ($CaO.SiO_2$)

1.2 Scaffold Used in Tissue Engineering Applications

The concept of replacing injured tissue or organs by biomaterial that are biocompatible, biosafe, non-allergenic, non-pyrogenic, non-teratogenic and so on has been in practice since the recent past. The selected biomaterial must meet the criteria of a non-toxic, effective, sterilizable material that is affordable for the majority of patients. A novel method that its concept was fabrication of a matrix or net which aids in providing a dynamic and complicated micro-environment for cells similar to ECM; they can grow, maintain, proliferate, migrate and differentiate. T.E was produced and rapidly industrialized which is shown in Figure 1.2.

Growing cells in vitro require special support and protection. Mechanical, physiochemical and biological properties should work in union to make a frame-scaffold-matrix-graft for different target cells as an alternative ECM. Biomimicking the natural ECM with growth factor assistance is used in remodeling tissue and replacement of traumatic or injured organs.

Scientists have been working on bone implantation (BI) for more than 50 years. Although the results were successful in majority of cases, there were some failures. So many articles have been published in this field since the first productive BI which was labeled by professor Ingvar Branemark-father of osseointegration- in 1960s. The purpose of this method is to

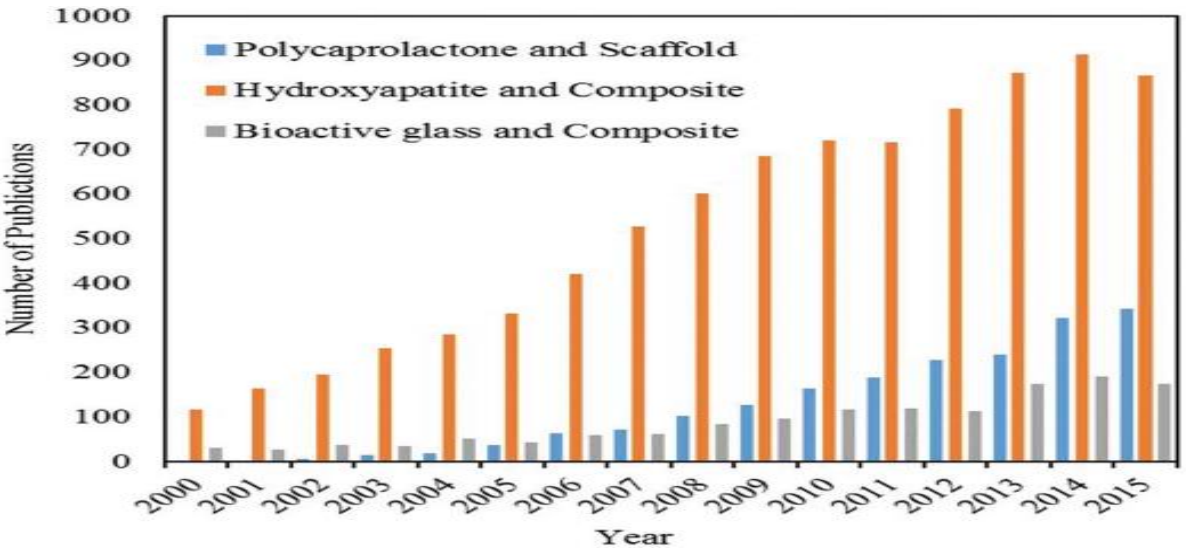


Figure 1.2: Number of the publications containing the keywords "polycaprolactone" and "scaffold", (FaezehHajiali et al., 2017)

preserve, improve and repair the function of tissue or organ. The use of scaffold in bone regeneration treats skeletal break or replacement and regeneration of lost bone. (Liliana Polo-Corrales et al.,2014)

1.3 Scaffold

In vivo ECM is a place which stores growth factors. G.F has two main function - the first is protecting the cell from degradation and the second it releases upon require. (Sònia Font Tellado et al., 2018) ECM proteins are composed of 90% collagenous mass (type I (97%) and type III, V, XI, XIII) and the rest 10% comprises of non-collagenous mass. Non-collagenous protein includes glycoproteins and proteoglycans. Glycoproteins contain alkaline phosphatase, osteopontin, bone sialoprotein and osteocalcin which controls the mineralization and modifies collagen fibril diameter. Other proteins are fibril, RGD integrin–binding domain (thrombospondins, vitronectin and fibronectin). Proteoglycans resists compressive forces from the glycosaminoglycan portion (long-chain polysaccharides) and binds growth factors (Liliana Polo-Corrales et al., 2014) so that the scaffold microstructures network is similar to ECM synthesizing as hydrogels, open-pore structures and fibrous matrices.

Scaffold is temporary frame that supports cells during cell proliferation, attachment and maintenance; this structure requires some properties. Three-dimension bulk has porous, vacancy or tiny chambers where cells are placed inside and channels that allow waste and nutrient pass through it. It is able to synthesize and analyze in vitro and later implant in vivo. It has different types based on either hard or soft tissue or in other hand it has variety of mechanical properties. Bioresorbable/biodegradable time is depending on molecular weight and mass loss of biomaterial that must be estimated to be equal to cell function. Another important factor is easy to manufacture with different size and formation. Lastly scaffold should be approved by FDA and GMP to be able to implant without any inflammation.

The four general categories of synthetic or natural in/organic biomaterials are shown in Figure 1.3.

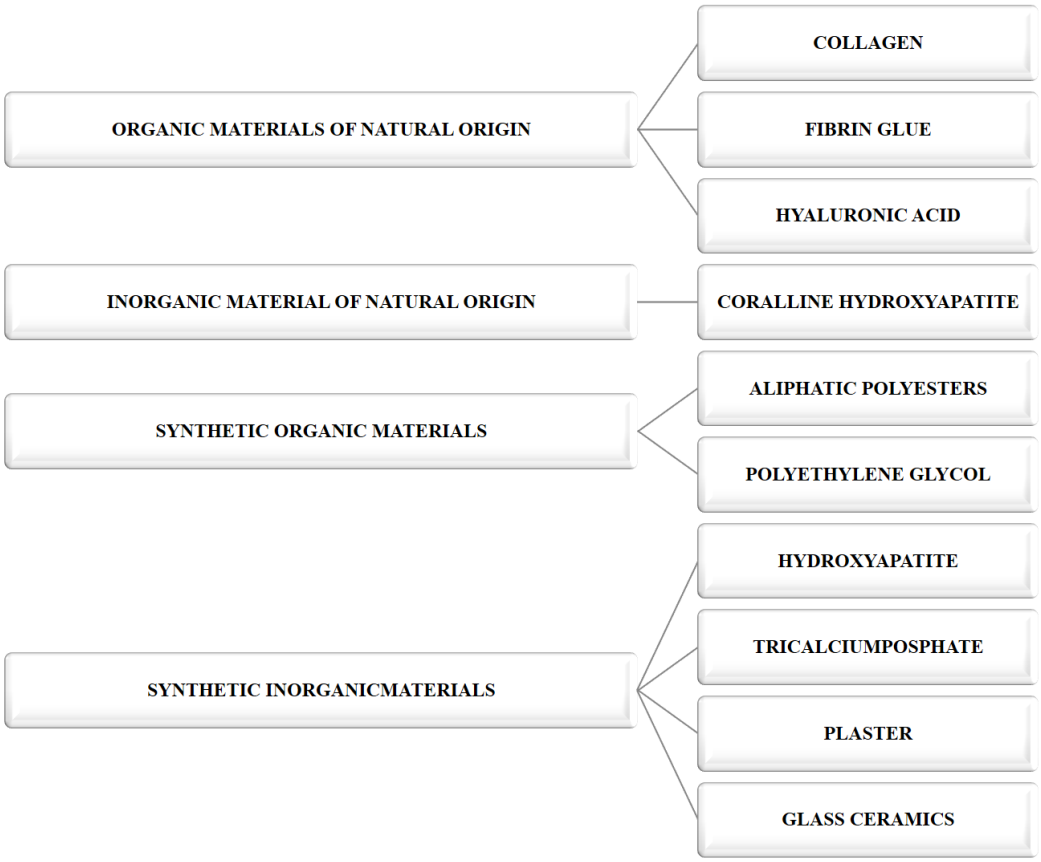


Figure 1.3: Scaffold material for tissue engineering application

Producing low molecular-weight compound may cause degradation fast and cause no leftover residue in the body. Figure 1.4 shows the MW, mass loss and duration of scaffold function for heart valve which is slowly dissolute in body-fluid and the result was without any cleavage by using solid polymer. Scaffold is mainly made of two types of polymers and bioceramic. The first one is biodegradable polymer such as polyglycolide (PGA), collagen, PLLA, alginate hydrogels, PCL and PDS. The second type is synthesis polymers like poly-lactic-acid-co-lysine. The factor that make specific scaffold for referred tissue is pore size for bone regeneration the porosity is estimated between 400 to 900 µm (Dietmarhutmacher et al.,2000).

Bioceramics can be classified by hydroxyapatite (HA) / Tricalcium phosphate (TCP) but fragility of these material is one of the disadvantages. Although the scaffold transmits loads and forces and should provide support, it does not necessarily have complete evidence to the mechanics of the tissue. The process of remodeling may differ among tissues. For bones it takes around 4-6 months depending on the host physiology and anatomy. There are many techniques for scaffold fabrication. Some of them, as mentioned in Table 1.2, are textile-technique (highly porous capability-fibers- polymers: PGA-PLLA, PGA, PGA/PDLA), particulate leaching, solvent casting, melt molding and membrane-lamination are the main techniques which are currently in use. Although some of these techniques have advantages, they have numerous disadvantages. The drawbacks are to be toxic solvents, require days or weeks for producing, have difficulty in processing and the requirements of specific equipment, retain complexity in controlling the size and shape to make thin and small structures because thick scaffold causes distance between the surface and leaves residual particles in structure, have non-regular pores and is insufficient and brittle. (Dietmar W. et al., 2001). On the other hand, advantages of fabrication of scaffold include, elimination of risk of disease and infection in transmission from donor and decrease in the number of operations.

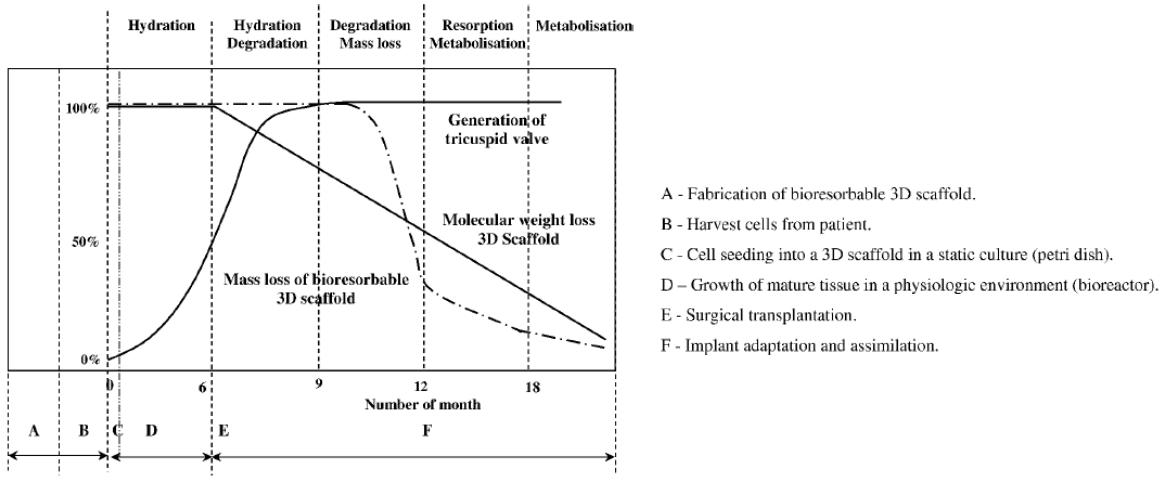


Figure 1.4: Mw and mass loss of the 3D scaffold cell/ tissue generation (heart valve) (D. W. Hutmacher, 2012)

Table 1.2: Scaffold fabrication technique (Tissue Engineering By Jan De Boer et al.)

Technique	Pore size(μm)	Biomaterial	T.E app	Reference
✓ Conventional Techniques	20-1000	Starch/SEVA-C	Bone tissue	Gomes et al.,2004;
✓ Foaming Using $CaCO_3$	100-1000	Chitin	---	Chow, khor,2000
✓ Sintered Microspheres	67-300	PLGA	Osteoblast/ Bone tissue	Borden et al., 2002
✓ Fused Salt Particles /Gas Foaming +Particulate Leaching	≤78	PLGA	---	Murphy et al., 2002
✓ Solvent Casting +Particulate Leaching	≤200	PLA/PVA	---	Mooney et al., 1995
✓ Solvent Merging + Particulate Leaching	250-500	PLGA	---	Liao et al., 2002
✓ Solvent Casting + Extrusion	≤30	PLGA-PLLA	---	Widmer et al., 1998
✓ Solid-Liquid Phase Separation (Freeze-Drying)	≤500	PLA,PLLA/ PLGA/PLA,HA	Osteosarcoma	Liu et al., 1992;Lo et al., 1996; Zhang and Ma ,1999/2001
✓ Liquid-Liquid Phase Separation	20	PHEMA/MMA	Neural and Spinal cord T.E	Dalton et al.,2002
✓ Polymerization	5-50	PHEMA/ PHPMA	Neural T.E	Hicks et al., 1999- Bakshi et al.,2004
✓ Emulsion Freeze Drying	≤200	PLGA/PEG	Bone T.E	Whang et al.,1995-1998
✓ Indirect SFF –Negative Mould Casting	50-800	PLA/HA,PPF/TCP	Bone T.E	Lin et al.,2005
	50-400	HA	Bone T.E	Wilson et al., 2004
	Polymer:1300 BCP:360	PEGT/PBT+BCP	Cartilage/ T.E osteochondral	Moroniet al., 2006

1.4 Films

Another well-known product in T.E since 1960 is modern surface or film. The simplest format of biomaterials is planar structure via different methods like casting, coating, spraying, covalent conjugation, polymerization, electrostatic and hydrogel binding. The units Micron to nanometer were fashioned to design lamellar structure, biosensor and drug delivery in the late 20^{th} century. Designing the film and modification of its surface played an important role in cell and tissue in medical fields. Surface patterned with bioactive addition is used for increasing functionalization. Due to their physiochemical properties they can be utilized on different surfaces for conductivity, wettability, reactivity and corrosion. The most essential conditions in thin film utility for biomedical application are its nontoxicity and its indifference on cellular functions.

Thin film is utilized on surfaces as a biologically active layer which then can reproduce on in the living body. The main properties of thin film are biocompatibility, versatility and biodegradability, can match to hard and soft tissue that have hardness, fracture stress, elasticity and young's modulus. Table 1.3 is shows the different method to make film among tissue engineering for various aspects.

Table 1.3: Synthesize film technique

	Technique	Process	Reference
Thin Film	Spin Coating Uniform Film On Flat Surface	Deposition Spin-up Spin-off Evaporation	Mozafari et al. 2016
	Layer-By-Layer Assembly (Figure 1.5)	Langmuir-Blodgett deposition* Dipping, Dewetting, Roll-to-roll, Centrifugation, Creaming, Calculated Saturation, Immobilization, Spinning, High gravity spraying, Atomization, Electrodeposition, Magnetic assembly, Electro-coupling, Filtration, Fluidics, Fluidized beds.	Iler RK,1966; Shim BS et al., 2007; Fujimoto K et al., 2005; Donath E et al., 1997;Grigoriev DO et al., 2008
	Dip-Coating Hydroxyapatite Dip-Coating Magnetic Scaffolds Bioactive Glasseceramics Biodegradable Materials	Immersion Startup Deposition Drainage Evaporation	Scriven LE,1988
	Electrophoretic Deposition	Not expensive and flexible coating process on metallic substrate surface, variety of thickness from nano to micro range. The main drawback is weak adhesion	M. Mozafari et al., 2016
	Biomimetic Approach	Forming thin film on surface of metals and polymers by using HA, bioactive glass and glass-ceramic specifically in bone defect	M. Mozafari et al., 2016
	Chemical Vapor Deposition	Under less than 1 atm pressure, some type of materials can form as film, the most advantages are low friction coefficient, fracture toughness, hardness and …	M. Mozafari et al., 2016
	Pulsed Laser Deposition	Depositing on surface with laser operating at 10 Hz , 380℃ for 1 hour.	
	Sol-gel Technique	Having more advantage compare to other methods, simplicity and high homogeneity. Classified into two film form:1-Inorganic oxide. 2- Organic and inorganic hybrid	Wang D et al., 2009

*One or more monolayers of biomaterial, liquid onto a solid by immersing or the solid substrate into the liquid. The disadvantages of this method are the high cost of instrumentation and consuming time.

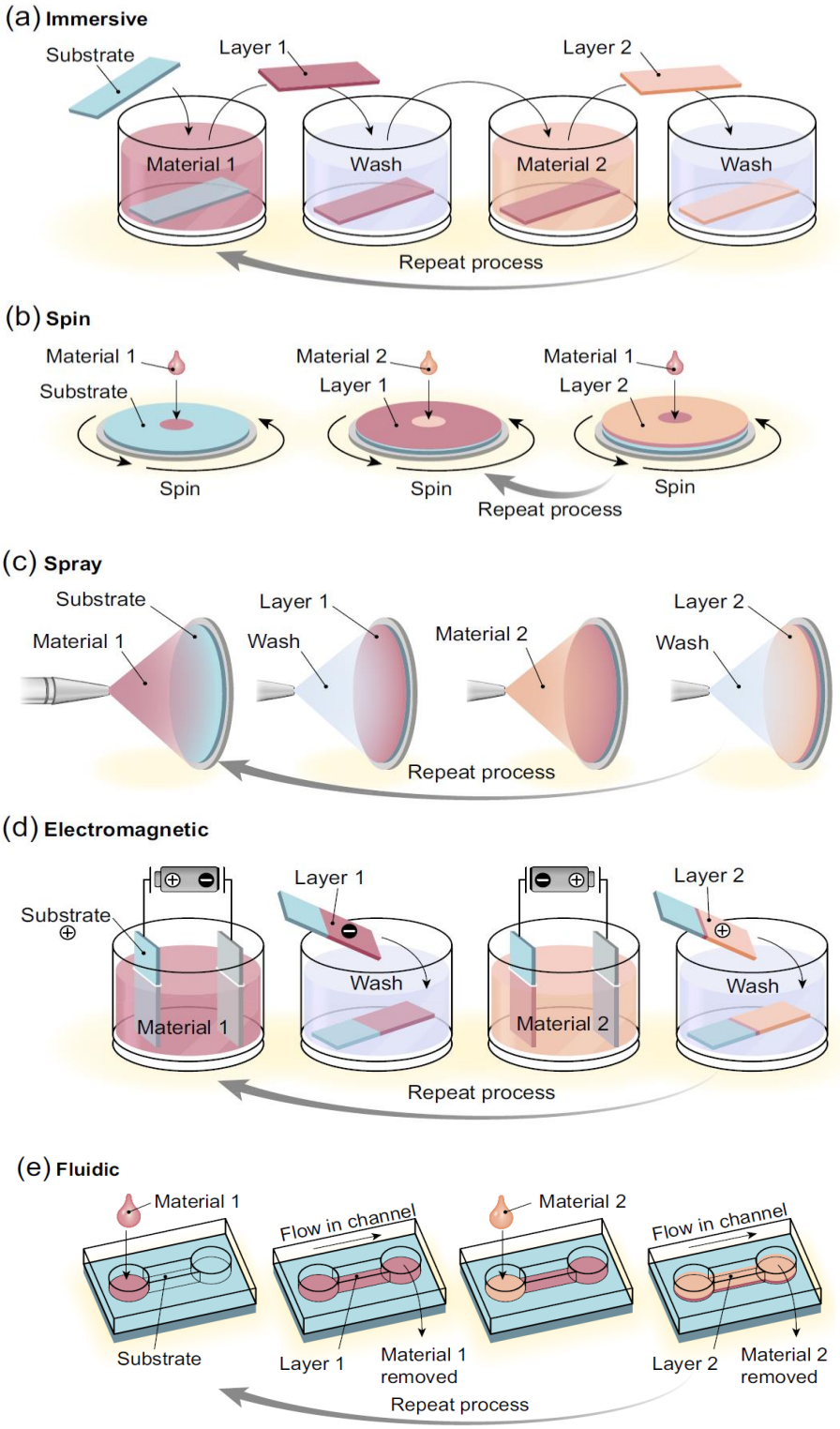


Figure 1.5: Film formation-Layer-by-layer assembly technologies (M. Mozafari et al., 2016)

1.5 Silk Fibroin

Natural Protein that is mainly extracted from silk-worms is called silk fibroin It is used in many biomedical applications due to its mechanical structure, biocompatibility and it is similar to normal tissue. S.F is used in medicine, textile industry and mostly in T.E due to its defined properties. It is also used in different form of sponges, mats, films, gels and fibers. For mechanical change and transforming S.F from α-helix into β-sheet by immersing in ethanol and methanol makes it insoluble in H_2O.

Although permeability of O_2 and H_2O and the mechanical-chemical-morphology of S.F prove it to be a suitable biomaterial, being highly brittle, having low flexibility and lack of hydrophilicity and osteoconductivity are the main disadvantages of its use. (Mehrnaz Moaddab, et al., 2018)

The chemical formula of amino acid is a mechanically highly elastic strong semi / crystalline structure. It can be degraded by enzyme or HFIP solution. Depending on the water temperature and the methanol treatment, S.F is highly conductive in most temperature controlled aqueous environments.

1.6 Chitosan

Cs powder ($C_{56}H_{103}N_9O_{39}$), Figure 1.6, is extracted from chitin and can be found in shellfish, crabs, shrimp or other sea crustaceans and fungi cell walls. This linear polysaccharide (β-1,-D-glucosamine and N-acetyl-D-glucosamine) is used in different fields such as food, cosmetic, cell culture and medicine. Negatively charged due to its CH_2OH (OH^-) groups, it can be excellent adsorbent in water and therefore can separate hydrophobic materials such as protein and metal ions. Its high biocompatibility, low toxicity, antimicrobial and biodegradability in biological system make it an important biomaterial in medical application. Its application can be classified as following:

1) Film
2) Hydrogel
3) Blocks
4) Tube

5) Sheets
6) Pellets
7) Sponge

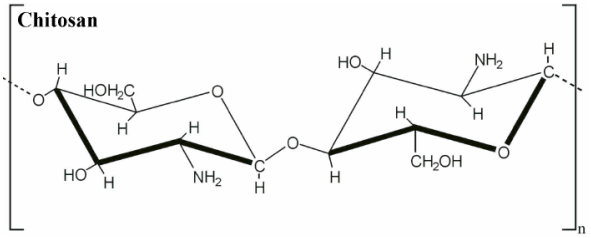


Figure 1.6: Chitosan structure. (García et al., 2014)

Another common name of this carbohydrate polymer is poliglusam. Range of molecular weight is between 300 kDa to over 1000 kDa that is mostly used in vitro. This natural biomaterial is utilized in T.E for replacing an injured area. It considerably effects fatty acid absorption, film forming and protein aggregation. Depending on the deacetylation degree, Cs properties will alter; for example, lower degree will lead to lower antimicrobial function. Cs has a similar composite structure as glycosaminoglycans (GAGs)- component in ECM in bone.

Cs has high use in antibacterial activity such as film and scaffold to protect against pathogen and microorganisms like fungi and bacteria. One disadvantage of Cs film is does not have surface porosity. For better surface treatment, the silica gel is a better choice but it is expensive therefore usage of this method has its limitation. PEG is another material, it is cheap and can control the pore size. Freeze drying in case of availability is another technique. Forming the film is the most common usage of Cs based on polysaccharides lipid, protein and resin. Cs has ability to incorporate with vitamin and mineral substances. In some cases, without any crosslinking or plasticizers; cellulosic, acrylic- co polymers and antimicrobial can make covalent attachment. At the range of PH 6 Cs will perform better at solubility and antimicrobial activity. Having an amino group that is positively charged, Cs is able to interact with negatively charged microorganisms or other biomaterials. Monosaccharide (CH_2O) is connected by covalent bond to O-glycosidic for making carbohydrate polymers

as polysaccharides. The acetyl groups contribute to form as hydrophobic micropores. (Luciano et al., 2013)

Basic polysaccharides like chitosan has viscosity, tendency to form biofilm and ability to dissolve in most medium yet somehow there is one disadvantage. Binding to microbial cell and mammalian make the bone structured since it is a binding agent.

Same as cellulose, Cs is quite highly insoluble and has a lower chemical reaction. Having 6.89% of nitrogen in its structure make it high commercial attention. This heteropolymer improves solubility in organic solvents. Cs is soluble in aqueous acetic environment and the concentration of Cs leads to different charge distribution which in turn effects the agglomeration.

The vast presence of hydrogen bond in Cs makes it degradable before its melting point to facilitate dissolving in a solvent. Some parameters are important for any solvent such as PH, concentration and temperature effects on solution viscosity.

Microfibrillar Embedding in protein network is one of the derivative formation of Cs. In 1926 the first form of ropy-plastic was made by using inorganic salts (CaI_2, $CaCl_2$, $CaBr_2$, $Ca(CNS)_2$) that have high hydration. In adding better dissolution, the first procedure involved 5% caustic-soda at 60℃ for 14 days, the second was for 3 hours at 180℃ and 10 atmosphere pressure and the best way was found in acetic acid solution at room temperature. It also has a poor osteoblast proliferation and bioactivity. (P. Sangsanoh, et al., 2010)

Wound healing and differentiation in tissue growth culture are main sectors of chitin and Cs use because of their absorbable properties. Further uses of Cs include removing heavy metal, film forming in flat dish at room temperature, optical characteristic such as lens-gas permeability towards oxygen, resistance to abrasion, using in cosmetic application such as lotion, cream and nail polish, drug delivery based on antacid and antiulcer activities to help lower drug interaction, swelling in acidic environment to cause a unique substance for drug delivery and hydrogel form because of its ability to swell. Similar structure of Cs to glycosaminoglycansis is used for skin replacement. Physical requirements like elongation, tensile strength, water content tear strength and modulus and oxygen permeability are the

most important properties. Table 1.4 shows different applications and the properties of Cs film. Figure 1.7 shows the polysaccharide and lipid that cover the surface of Cs which can be use in different aspects such as drug delivery because of its swelling behavior. Figure 1.8 and Figure 1.9 shows ionic bond between Cs and alginate in different PH. The suitable cross linker used mostly in papers is TPP and its properties make the product flexible and help increase its stability. (Chunxiu Liu, et al. 2004)

Table 1.4: Film application of Cs

#	Application	Properties	Reference
1.	Photography	Abrasion-resistance Optical characteristic Film forming ability	R.A.A. Muzzarelliet al., 1997
2.	Cosmetics	Fungicidal and fungi static Natural cationic gum-viscous on being neutralized with acid (creams, lotions)	H.F. Mark et al., 1985
3.	Artificial skin	Structural characteristics similar to glycosaminoglycans for skin replacement	Y. Le, S.C et al., 1996; D.A. Sandford et al., 1991
4.	Based dressings	Wound healing products Treating plastic surgery Chitin-based fibrous dressings	Kanke M et al., 1986. Hirano S et al. 1989. Hirano S et al., 1999.
5.	Food and nutrition	Utilization of whey to improve animals nutritional	Rinaudo M et al.,1999.
6.	Opthalmology	Ideal contact lens (clear, tough and other required physical properties) Optical clarity, mechanical stability, sufficient optical correction, gas permeability, particularly towards oxygen wettability and immunological compatibility.	Rinaudo M et al.,1999.
7.	Water engineering	Environmental protection Metal capture from wastewater Removal of mercury from solutions	Domszy JG et al., 1985.
8.	Colour removal from textile	Able to sorbet metals and surfactants Attract basic dyes and other moieties by increase in the temperature Low pH, chitosan's free amino groups are protonated causing them to attract anionic dyes	Pelletier A et al.,1990.
9.	Paper finishing	Impart wet strength to paper	Heux L et al., 2000.

10.	Solid-state batteries	Insoluble in water Provide ionic conductivity when dissolved in acetic acid The conductivity is due to the presence of protons from the acetic acid solution	Va˚rum KM et al., 1991.
11.	Drug-delivery systems	Controlled release dosage Safety, efficacy and reliability Biodegradability, slow and controllable diffusion Non-toxic and easily bioabsorbable with gel-forming ability at low PH. Chitosan has antacid and antiulcer activities which prevent or weaken drug irritation in the stomach Swell in an acid medium Drug release behaviour using silver sulfadiazine, Vitamin A, vitamin E and riboflavin as a model drug	Va˚rum KM et al., 1991-Rudall KM et al., 1973. Atkins EDT et al., 1985.
12.	Hydrogels	Hydrogels have been widely used in controlled-release systems Highly swollen, hydrophilic polymer networks Absorb large amounts of water and drastically increase in volume Directly compressed tablets Chitosan/potato starch Crosslinked chitosan microspheres coated with polysaccharides or lipid Microcapsules/microspheres of chitosan Chitosan/gelatin network polymer microspheres Chitosan microspheres for controlled release of diclofenac sodium Chitosan–polyethylene oxide nanoparticles as protein carriers Chitosan/calcium alginate beads Multiporous beads of chitosan	Marguerite Rinaudo, 2006

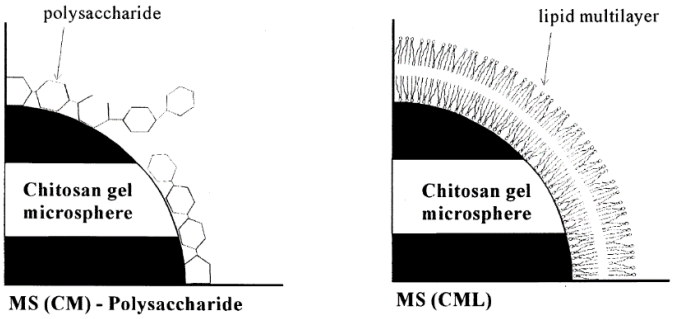


Figure 1.7: Chitosan gel - microsphere coated with anionic polysaccharide and lipid (Majeti N.V. Ravi Kumar, 2000)

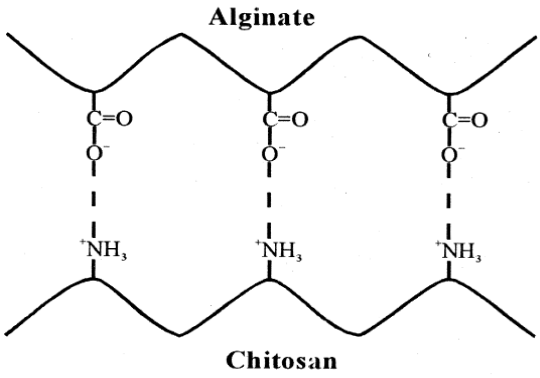


Figure 1.8: Ionic interactions between PH=5.4 (Majeti N.V. Ravi Kumar, 2000)

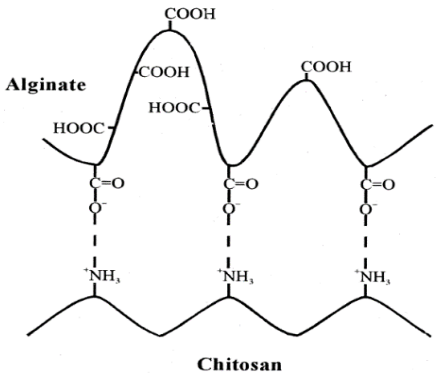


Figure 1.9: Ionic interactions between PH=2.0 (Majeti N.V. Ravi Kumar, 2000)

1.7 Eggshell

One of the economic resources and mineral hazardous waste is eggshell; ES and ES structure are shown in Figure 1.10 and Figure 1.11. Poultry eggs have approximately 10 percent solid shell. E.S is natural waste that is formed in the final stage of poultry oviduct. Due to mineralized structure it is the best polycrystalline bioceramic. Its compounds are similar to skeletal system used in bone tissue regeneration such as 96 %calcium carbonate and 1 % Magnesium carbonate, 1 % calcium phosphate and the rest are organic material- protein based and water.

Figure 1.10: Eggshell

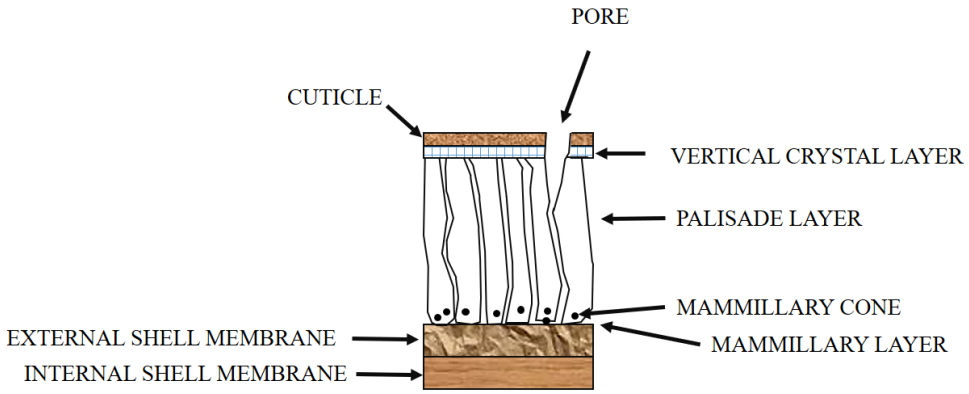


Figure 1.11: Eggshell structure

Thousands of researchers have investigated different biomaterial which their function and structure are similar to ECM. Providing mechanical, physical and chemical platform for cell-culture. So that the combination of ECM and cell definition are unique characteristics of

tissue. Regulating the cellular performance, interacting and signaling with growth factor and molecules are central responsibilities for ECM. ECM is a protein that is made of glycoprotein, collagen, elastin and proteoglycans. Natural polymers are the main group that play important role such as chitosan, hyaluronic-acid, silk fibroin and fibrin glue. (Mahesh Kumar Sah, et al.,2015) HA is a mineral bioceramic that has been used in a variety of application because of its similarity to the human bone. Avian's egg are closed meshwork to HA bioceramic. Mechanical strength, bone bonding osteoconductivity of HA are good properties for synthesizing of hard tissue like bone, and the best PH range due to stability is at 4-12, surface modification of ES can perform in polymers, protein, acids, natural polysaccharide, silane, peptide and alcohols. Having P-OH groups (hydrophilic) in surface of HA make better adsorption for water molecules and CH_3OH. (Siti Maisurah Zakaria et al., 2012)

Having excellent mechanical properties of HA, ceramics and glass (included apatite and $CaO.SiO_2$) are use in bone defect as biomaterial and various type of applications, on the other hand they are not able to withstand high loading condition especially femoral and tibia bones, that they are under high pressure due to high elastic modulus and low fracture resistance (fracture toughness) Table 1.5 is shown properties of HA, α-TCP and β-TCP.

Table 1.5: Some typical properties of HA, α-TCP and β-TCP (Faezeh Hajiali et al., 2017)

Material	Chemical formula	Density (g/cm^3)	Melting Point (°C)	Solubility in water at 25°C (mg/L)	Compressive Strength (MPa)	Young's Modulus (GPa)
HA	$Ca_{10}(PO4)_6(OH)_2$	3.16	1614	0.00010	500–1000	80–110
α-TCP	α-$Ca_3(PO4)_2$	2.87	Liquifies under high pressure at 1391°C	0.97	—	103–188
β-TCP	β-$Ca_3(PO4)_2$	3.07	Liquifies under high pressure at 1391°C	0.20	460–687	33–90

Increasing ionic activity in body fluid, hydration of silica on surface are caused by releasing calcium from apatite and bioactive biomaterial, then make sites of apatite nuclei. So that by getting calcium and phosphate ion, this apatite will grow and create layer of bonelike.

ES and its membrane have more than 500 proteins (X-collagen, Ovocalyxin-21, Serum albumin, Lysozyme C, Cystatin, Clusterin and Calcyclin …) in their structure, that 4 or 5

times more than egg white and yolk and proteoglycans such as keratin sulfate proteoglycan, mammillan, ovoglycan. ES can be diluted in acetic acid, HCl and EDTA. ES is formed by the carbonic anhydrase enzyme and the role of eggshell membrane- non mineral layer. Mainly egg white components, ubiquitous components and components unique to eggshell are 3 groups of molecules which form ES. Bi-layered fiber membrane and calcified matrix were shaped within 22 hours at T≤ 40℃ through oviduct. Crystallized 5 gr of calcium carbonate in 20 hours to create the ES is one of the fastest bio mineralizing systems.

1.8 Glycerin

Glycerin Figure 1.12 is nontoxic to human health, safest materials in industrial chemical and is used in so many areas.it is hydrophilic, soluble in cold or hot water or in alcohol, colorless, viscous liquid, melting point is at 18°C,attract moisture and boiling point is at 290 °C all as physical and trihydric alcohol or sugar alcohol (75% as sweet as sucrose) as chemical properties. Glycerin was accidentally found in 1779 by K. W. Scheele while he was heating a mixture of olive oil and litharge. Molecular Weight of G is 92.09. Glycerin is used as a thin film for making soften textile or to become like glue or gelatin. Alkyd produced from glycerin is an important class of resins used in surface coatings.

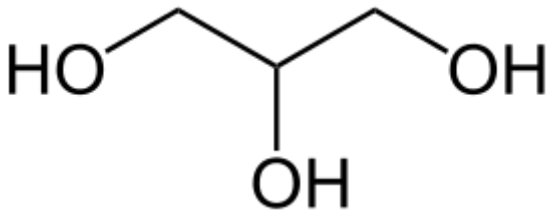


Figure 1.12: Glycerol structure

1.9 Aim of Study

Synthesizing film and scaffold from three main biomaterials for bone regeneration. Using chitosan, silk fibroin, eggshell and glycerin for fabricate natural meshwork. Bone remodeling with osteoblast in vitro requires controlled parameters and the most important is

fabricating form of graft that its structure is closed to bone tissue, low cost, easy to make and biocompatible with biological system

1.10 Literature Review

HA/Cs gelation form was introduced by Feng Zhao et al. in 2002. They claim this composition is similar to the human bone and they prepared 3D scaffold by neonatal rat caldaria osteoblasts with 90.6% porosity. Make up about 70% of bone structure and osteoblast was securely attached to surface and degradation and swollen was successfully occurred as but in paper did not mention any drawback. (Feng Zhao et al., 2002).

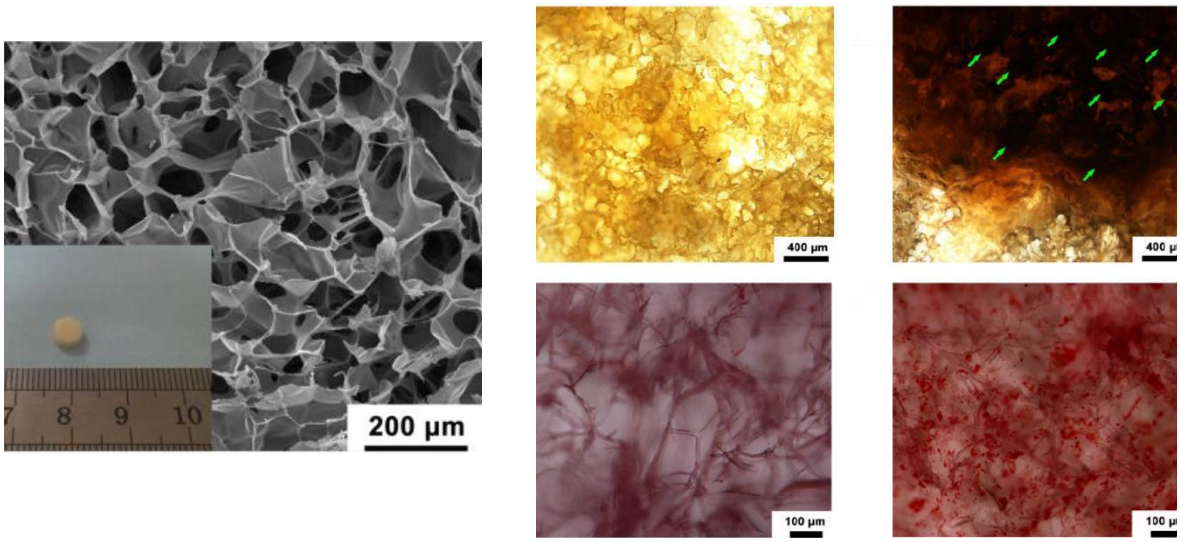


Figure 1.13: Scaffold and cell culture Cs/S.F (Da-Wei Li et al.,2017)

3D scaffold was prepared by Chitosan/silk fibroin to mimic natural ECM shown in Figure 1.13. Freeze-drying method was used to make this scaffold and seeded by rat cell and implanted in vivo. 2-4 weeks after implanting the scaffold was successfully showed excellent cytocompatability without ant inflammatory cell and newly blood vessels was generated. 89.4 ± 4.2% of porosity and pore range was between 86 -133 µm,39.8 ± 1.8 KPa and 10.2 ± 0.8 KPa are compressive modulus and strength respectively. (Da-Wei Li et al.,2017) Cs/Alginate 13mm in diameter and 12mm in thickness scaffold was fabricated. (Zhensheng

Li et al., 2004) Cs as cationic and alginate as anionic polymers solution maintained by lyophilized in a freeze dryer method then cross linking with $CaCl_2$.

Cs with Sodium tripolyphosphate as a cross-linker and the density of TPP increases, the hydrophilic and hydrophobic nature of the micropores increases. (Luciano et al., 2013)

Bioactive bone regeneration scaffolds is made of SF-carboxymethyl chitosan with chitosan nanoparticles encapsulated ascorbic acid. Improving in osteoconductivity and osteoinductivity of SF scaffolds occurs by combining of AA and CMCh. (Mehrnaz Moaddab et al., 2018)

Fabricating encapsulation of vitamin C by using Cs as encapsulating, tripolyphosphate as crosslinker and spray drying was the method, classifying the carbohydrate polymer is in Table 1.6. (Ramesh Murugesan, Valérie Orsat, 2012)

Table 1.6: Materials and their sources (Wandrey et al., 2010)

Origin	Carbohydrate polymer	Protein	Lipid
Plant	Starch	Gluten (corn)	Fatty acids/alcohols
	– Derivatives	Isolates (pea, soy)	Glycerides
	Cellulose		Waxes
	– Derivatives		Phospholipids
	Plant exudates		
	– Gum arabic		
	– Gum karaya		
	– Mesquite gum		
	Plant extracts		
	– Galactomannans		
	– Soluble soybean		
	Polysaccharides		
Marine	Carrageenan		
	Alginate		
Microbial/animal	Xanthan	Caseins	Fatty acids/alcohols
	Gellan	Whey proteins	Glycerides
	Dextran	Gelatin	Waxes
	Chitosan		Phospholipids (Shellac)

1.11 Osteology

The bone is an organ with a complex three-dimensional microstructure and two main types: spongy (trabecular or cancellous bone) and compact (cortical). Its function is supporting, protecting other organs and cooperating with joint for movement. Osteo tissue is source of mineralized materials approximately 97% calcium whenever there is a need it can be distributed into the target tissue distinct from marine animals that calcium can be obtained from their ingested food so that the amount will be limited. It can produce red and white blood cells in its marrow as well.

Bone can be simulating as steel frame in building which tolerates the load and forces. In 1987 Frost said about the theory "Mechanostat" about controlling the bone mass in response to mechanical loading but it was not clearly proved so that later on the research was calculating the force using lumber vertebral bodies of rat in 1993 by Harrigtonetal. Basic biomechanical measurement of bone by C.H Turner in 1993 via CAD strategies was introduced although it was presented description of several test, because of variety of bone sizes was incomplete. Stress or force per unit area classified as compressive makes the material become shorter, tensile when the material is stretched and shear through the cross section of material and slide over another. Strain is an extension per unit length or relative deformation. So that the micro and macro-structure effects its mechanical properties. Elasticity property of bones can lead to the regains its original shape and size by variety of stress and not to be brittle. Another important factor is bone mineral density. Low bone density leads to osteoporosis disease. Measuring bone density can be done by Absorptiometry, QCT, QMRI, US in main three areas –the hip –spine –total body.

Defection of bone structure is a problematic issue which auto graft is one of the desired method but has difficulties for collecting, may occur infection and harm for its function. It has been used for regenerating tissues, harvesting few amount of cells from patient and culturing in sitro till desired amount of cell is produced; transfer them into premade scaffold and finally placed in vivo or defected area. As a cell proliferation arises, scaffold will be degrading and as a result uniform and natural tissue will be formed. (Robert C. et al., 1998)

So that the best way to overcome challenges is usage of appropriate scaffold either it is made of natural or synthetic biocompatible biomaterial. On the other hand, scaffold should have the specific ability to be resorbable as well as biodegradable in certain duration, equivalent for cell culture that regenerate tissue. (Herve Petite et al., 2000) scaffold has certain parameters such as a vacancy where cell is placed and also for transporting of nutrition and waste; this interconnection 3-dimensional system with its highly porous capacity requires proper surface chemistry for cell attachment and its function (Dietmar W. et al 2000); Mechanical properties of this network is another contest to establish the structure and support cell.

Several biomaterials have been presented for bone and cartilage scaffold for instant HA as short fibers or tricalcium phosphate, PHE and natural polymers are used to form this frame likewise they are approved by FDA. (Robert C. et al.,1998)

PLGA foam has been used in load-bearing App because of their compressive strength. (Thomson RC. et al., 1995)

Composition of polyacrylic acid, polysaccharide, bioceramic and other biomaterials assist in healing by improving osteocunductivity and constructing the defect site. Using particulates and fibers materials have yielded promising results with improve strength and mechanical properties respectively. (Gillet . et al., 1985 – Marom G. et al.,1993)

HA/PHE was introduced in 1998 as a scaffold for bone and cartilage regeneration. Degradable by osteoclast, increasing the speed of renewal bone and large pore by producing foams (short fibers) were their goal to design this composite system. PLGA and Lactic acid (LA) were used. 8-12 weeks are estimated time of healing the defect but might be vary.

Designing 3 dimension printed bioactive based on ceramic (3DPBC) included HA and β-Tri-calcium phosphate (βTCP) for bone defect such as trauma or infection. The scaffold was coated with bone-morphogenetic-protein (BMP-2), saline or dipyridamole. For activation of osteogenic via decreasing osteoclast and increasing osteoblast by receptor that is called adenosine A2A (A2AR) and the reason of this activation was interaction of dipyridamole with the specific A2AR through ligation of A2A. Scaffolds were placed in

C57B6 for 2-8 weeks and as a result showed increasing bone formation. (Wiley Periodicals et al., 2015)

HA, CS, CP, DBM and BMP are some of the growth factors with MSC and other agents are utilized to form bone injuries. Although they have advantage to use in scaffold, may occur cancers later on that can be one of drawback specially BMP-2. (Stephanie Ishack et al., 2015)

Vertebral fragile of bone can be reduced by using Sr^{+2} Ion (Strontium ions). It aids bone formation and prevents bone resorption recently was considered among researchers. They are able to activate osteogenic. The scaffold made of collagen & Sr^{+2}& HA and CaR as a receptor. MARK makes MSC differentiation process in vitro. (Fan Yang. et al., 2011) Wnt is protein attached to palmitoleic acid and there are 19 different gens in mammals and they are known as signal pathway. Wnt/β-catenin which helps to develop embryonic so that any mutation can occur cancer. The most important fact presenting Wnt/β-catenin leads to raise growth and proliferation and progressing stem cell to osteoblast. (Clevers H et al., 2006) (Chen Y. et al., 2007) Sr^{+2}ion can active Wnt/β-catenin and help bone regeneration. (Fan Yang. et al., 2011)

Substitution are classified into natural and synthesis. Polymers, metal, CaP and corals are naturally or from agricultural waste used in different scaffold and bone grafts and bioactive glasses based on Si. Closeness properties of these material to bone structure is CaP. This composite has ability to dissolve, degrade, cell interaction leads cell to be function, bioactive to form bone, osteocunductive,

Some of investigation were mentioned above. Skeleton scaffold is key to as a temporary support placed in lost tissue. The most broadly studied about a common biomaterial like calcium-phosphate, it is combines with HA or TCP has crystal structure and can classified as polycrystalline ceramic. Permeable structured pore and organized might be advantage that can extract from variety of resources and have pure HA to produce calcium-phosphate-ceramics.

Mineralized ECM of bone cell and calcified tissue mixture of organic (non/collagen protein) and inorganic (mineral). bone mineral phase is almost calcium hydroxyapatite. It is

carbonate-hydroxyapatite and its formula is $(Ca,A)_{10}(PO_4,HPO_4,CO_3)_6(OH,B)_2$; the A element (a substitute of Ca ion) is a cation (Mg,Na,Sr ions) and B element (a substitute of hydroxyl group)is a anion (Cl, F ions). (Le Geros. et al., 2002)

Since fetal life, bone modeling will start till the highest level, during adolescence, it will be remodeled by biomechanical forces. It assists to renew old and damaged bone as a result replacement by stronger structure. This process will continue to reach to peak level of bone mass, growth and density. Genetic, hormones, physical activity and nutrition have effect on quality of bone and lead to microarchitecture of bone tissue.

Long, short, flat and irregular (Table 1.7) are four classified bones by size and shape.

Table 1.7: Bone Type

Bone type	Example
Long	Clavicles - Humeri - Radii - Ulnae - Metacarpals - Femurs - Tibiae - Fibulae - Metatarsals - Phalanges
Short	Carpal - Tarsal - Patellae - Sesamoid
Flat	Skull - Sternum - Ribs Pelvis
Irregular	Vertebrae - Sacrum - Coccyx - Hyoid

As mentioned in introduction skeletal system is the hardest connective and self-renewable tissue in human body. This hardness is due to inorganic salts that make 60 % of the total bone mass. These salts are Calcium, Phosphate, Magnesium, Carbonate, Sodium and Fluoride. Lack of calcium makes the bone structure soft and can bend easily. Collagen type (I) is a kind of central nuclear which mineral salt can sediment on it. As a result, HA a bioceramic structure will be made. It provides structural support, allows body to move or a place for muscle attachment, protects internal organs such as brain and bone marrow and storage of mineral material. Formation, modeling and remodeling of bone are based on three cells. Osteoblast as forming cell, osteoclast as resorbing cell and numerous osteocytes – ten times more than osteoblast- (Parfitt, 1977). There are so many assumptions related to osteocytes. overtimes researchers believed that osteocytes could remodel their ECM, remodel bone, be mechanosensory cells. Osteocyte is in bone matrix located enclosed within

lacuna, from MSC through osteoblast differentiation. When osteoblast is stablishing bone matrix, some becomes surrounded in matrix first in osteoid area – non-mineralized zone – after while by calcification of osteoid a mature osteocyte will be formed and become stellate shaped by the time osteoclast during bone resorption will destroy osteocytes or when the matrix will degrade the liberated osteocyte from lacuna and differentiate into fibroblast (osteoblast). Osteocytes and canaliculi are the only communication way to outside. Nutrition and waste product will be transformed to and from blood circulation by this cell and its canals. (Palumbo et al., 1990) intercellular attachment sites where osteocytes connect with neighboring cell therefore it creates a complex network. Lytic enzymes (collagenase, acid phosphatase and aminopeptidase) are presented in osteocytes. (Woods and Nichols, 1965) (Doty et al, 1968 and Lipp, 1959) Its function is machanosensor of local strain in bone, meaning that as a result of loading osteocytes are sensitive to mechanical forces so that their activity will be increased by extracting G6PD. They are inactive until fracture occur on bone, they will divide into osteoblasts and ancestral cells.

Bone formation depends on osteoblast cuboidal-shape, 4 to 6 percent of the cellular content. (Mattia Capulli, 2014) Its function is to produce new bone matrix by synthesis of ECM or hydroxyapatite crystal in graft. By following Secretion of collagen type (I), non-collagen protein (Osteocalcin, Osteonectin, Bone Sialo Protein II and Osteopontin) and Proteoglycans (Decorin and Biglycan) the osteoid zone will be derived from membrane osteoblast membrane (W. Yang. 2005), afterward synthesis of HA crystal within graft that aid to fill

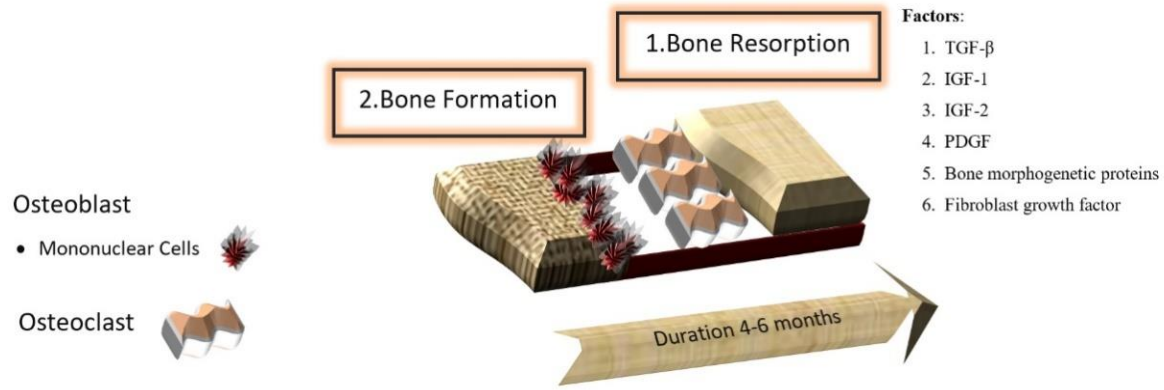


Figure 1.14: Bone formation

the gaps between collagen and fibers. Mineralization and non-mineralization bone can observe by von Kossa and van Giesonstaining. (Mattia Capulli, 2014)

Osteoclast is known as cell capable of destroying bone or bone resorbing derived from promonocytic precursors which they are in bone-marrow (macrophage CSF (M-CSF) and RANKL), spleen and peripheral blood. (Donald G.Walker et al., 2016). Figure 1.14 is represented as bone formation. Multinucleated cell by releasing proton and secretion of acid and Proteolytic enzymes (Carbonic Anhydrase II- vacuolar-type H+ ATPase (V-ATPase)-Cathepsin K- Tartrate-Resistant Acid Phosphatase (TRAP)) in EC to dissolve the mineral and collagen components. (R. Marcus, 2013) Compare to osteoblast, osteoclast process is more rapid in acidic environment. It is able to function in both oxygenated and hypoxic region, cell fusion, huge mitochondria and multinuclear make osteoclast specific in energy metabolism matter and mainly this principal energy sours is D-Glucose. (J.P. Williams et al., 1997)

Osteoclast produce ATP by Glycolysis (can convert glucose into pyruvate that is metabolized to CO_2-in present of O_2- or lactate -in absent of O_2-and releasing energy, otherwise) and Oxidative Phosphorylation.

Periosteum is a connective membrane on bone surface that contains blood vessel canals as volkman's canals, nerve networks, nutrition and cells (osteoblast and osteoclast) for growth and repairing the bone fracture. Periosteum consists of two layers, outer (fibrous) and inner (osteogenic) and by aging the surface will increase.

Bone mostly appear in form of cartilage during first stage of life. By the time it will transform into bone. Dense/solid bone which surrounds the bone marrow. Longitude and circular of bone will increase and remodeling takes place by changing shape while mechanical forces applied on bone. Some mechanical properties are classified in Table 1.8. Activating osteoclast within 2-4 weeks for renovation. Porosity of bone tissue is about 1-100 μm but differ from trabecular which is around 200-400 μm. The pore size will be varying during life time by nutrition or all regeneration factors.

As a summery bone graft has three main processes:

1. Osteoinductive: form new bone and stimulate progenitor cells for differentiation into preosteoblasts to begin the bone-forming process.
2. Osteoconductive: supportive attachment of bone forming cell and stimulate cell to grow on scaffold surface.
3. Osteogenic: have cells that are involved in bone generation/formation such as osteoblasts, and osteocytes mesenchymal stem cells (MSCs)

Table 1.8: Mechanical properties of wet compact bone (Faezeh Hajiali et al. 2017)

Target Tissue		Porosity (%)	Density (g/cm^3)	Tensile Strength (MPa)	Compressive Strength (MPa)	Young's Modulus (GPa)
Bone	Cortical bone	5–30% (mostly 5–10%)	1.81	122.9–175.2	102.7–111.3	17.5–18.9
	Cancellous bone	30–90% (mostly 75–95%)				

1.12 Surface Modification

As mentioned in previous section bone matrix in included mainly type I collagen and the rest with types III, V and FACIT collagens that aids in certain stages of bone formation, these fibrillar and nonfibrillar collagen make ECM stable. Also it consists of 25% noncollagen protein such as HAs (assists mineralization) and α2-HS-glycoprotein (regulate proliferation of bone cell) that are attached to HA because of acidic properties; growth factors (BMPs, TGF-β, FGF, IGF, PDGF, interleukins) and other activated molecules are in noncollagen protein category. Cell attachment require specific protein some important protein which present in bone and help the bone function are glycosylate (alkaline phosphatase - its function is mineralization of bone), bone sialoprotein, proteoglycan, osteopontin, γ-carboxylated (gla) and osteocalcin,

Mineral contains 50-70 % of bone; HA (small crystalline protein with dimension ≤ 200 Å – support mineral metabolism), acid phosphate, carbonate and Mg are major composites of mineral. Organic matrix between 20 to 40 % and Water is approximately 5-10 % and less that 3 % is lipid construct bone structure.

All the calcium and phosphate aid to order statement of mineral by controlling quantity and size of HA crystals.

A suitable surface should provide mineral for mechanical and loading forces, have elasticity and bendable properties. Increasing acidic phosphate, inorganic phosphate and calcium can lead to crystal formation for mineralization of HA crystal. As bone grow the process of crystallization starts from amorphous calcium-phosphate to HA crystal.

The amino as hydroxyl groups in chitosan (glucosamine unit) structure are substituted by carboxymethyl ($-CH_3COOH$) presence of carboxyl (-COOH) groups in biomaterials helps osteoblasts adhesion and proliferation.

CHAPTER 2

MATERIAL AND METHOD

2.1 Experimental and Preparation

In this chapter fabrication of 3D scaffolds and films by different parameters will be described. The structure and other properties has been analyzed.

2.1.1 Material

Chicken eggs were purchased from North Cyprus. Chitosan (CS) (MW 500,000-190.000), $CaCl_2$, NaOH, Methanol, silk fibroin (S.F), Glycerin (G), acetone, acetic acid (AA) and Tripolyphosphate (TPP), ethanol and distilled water (DW) are materials which were used during this experiment.

2.1.2 Preparation of material

The chicken eggs were washed with pure water and liquid cleaner then break down, yolk and albumen were separated. The ES was cleaned with distilled water. While they were wet manually ES was separated from eggshell membrane (ESM). After separation all, the ES was dried in incubator for one hour and the temperature (T) was 70℃. ESM was kept in phosphate buffer solution, PH=7 in refrigerator until they were used.

After drying ESs, they were grinded. The powder size was 0.212 mm and kept into a plastic container.

The first method is described as Cs was measured 4.8 gr and mixed with AA and DW amount 34 ml and 150 ml respectively, it takes around 10 hours, T=65℃ with 1000 rpm to become homogenously as a solution.

In the second method Chitosan (MW 500,000-190.000), 4 wt% Cs (4 gr/solution) mixed with solution of acetic acid (0.175 N) (1.00 ml of acetic acid to 25 ml deionized water and adjusting the final volume of solution to 100 ml with deionized water). It takes around 3 hours, 5000 rpm to become homogenous.

PBS was made by NaCl 8.0 g, KCl 0.2 g, KH_2PO_4 0.2 g and 1.44 g of Na_2HPO_4 in 1 L of water.

S.F 2.5% was prepared. Firstly, cocoons were in 0.1 M Na_2CO_3 (1gr:100 ml) at 70℃ for 3 rounds, each 3 hours the silk was washed and solution was changed. Next step is drying silk fibroin at room temperature. Dissolution was the following and last step to break down the polypeptide chain by C_2H_5OH: H_2O: $CaCl_2$ (2:8:1 M) solution at 70℃.

$CaCl_2$ 2 M, NaOH 5 M and TPP 5% was made for crosslinking the Cs and adjusting the PH level of Cs correspondingly.

2.1.3 Preparation of scaffold

The first set of scaffold was made of ES powder. 0.5 grams was weighted and mixed with 1 ml Cs, (0.15 gr) of G, 100 µl of SF. The compound was kept in an incubator that was adjusted to temperature of 70℃ until the sample dried. The first 30 min while the sample was drying, covered by $CaCl_2$ for 3 min then covered by Methanol and put back into the incubator. This process was repeated one more time after 15 min and finally the sample was kept in the incubator to dry completely. The second Sample-Cs based- was made of 0.25 gr ESs following the same procedure. Different measurements were shown as in Table 2.8.

The second way the compound was added which is shown in Table 2.9; sample was made and kept in the incubator. Temperature was adjusted at 70℃ until the sample dried. Then the scaffold was crosslinked by TPP (5 %wv) for 5 min, 10 min, 15 min with each time being washed with pure water. Finally samples were washed by Ethanol:PBS (PH:7.4) 40%:60% v/v and removed and kept in a petri dish.

2.1.4 Preparation of film

The following films were fabricated same as the scaffold but with different thicknesses and with the addition of acetone and methanol treatment; samples are shown in Figure 2.15.

ES powder was measured 0.01, 0.005, 0.0025, 0.001 gr. First powders were treated by some drops of HCl. Then the following composites were equally added to each four ES, 1 ml Cs, 0.06 ml G and 100 µl SF. Then put on a magnetic stirrer at 70℃ until the sample dried. The

first 15 min while the sample was drying, covered by $CaCl_2$ for 3 min then covered by Methanol and acetone. Then, we remove it from the petri dish and keep them to dry; Table 2.9 is representing the amount.

In the second method, the film was made following a similar process as the scaffold but with a different amount of ES and 0.125 ml G. The composite was mixed and heated at 70℃ until the sample dried. Then, the layer was crosslinked by TPP (5 %wv) for 5 min, 10 min, 15 min each time washed with pure water. Finally samples were washed by Ethanol:PBS (PH:7.4) 40%:60% v/v and removed and kept into petri dish as shown in Table 2.10.

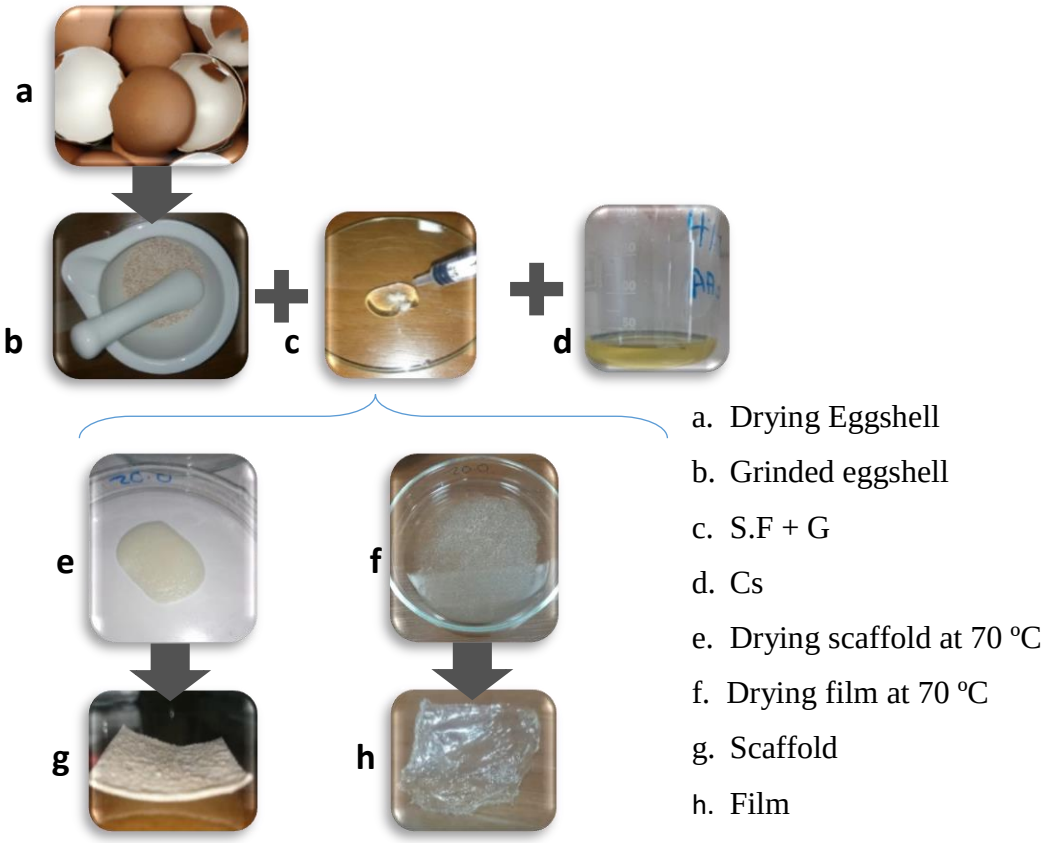


Figure 2.15: Scaffold and Film Procedure

Table 2.9: First method Scaffold and Film preparation

Method	Type		Eggshell (gr)	Chitosan (2%) (ml)	Glycerin	Silk Fibroin 75%	Picture
Leaching & direct dry method: ***1.*** *CS, G,S.F and eggshell is mixed* ***2.*** *T=70 C dried the layer* ***3.*** *Cover with* $cacl_2$ ***4.*** *Dry T=70 C* ***5.*** *Methanol poured on it* ***6.*** *dry T=70 C* ***7.*** *Step number 3 till 6 repeated one more time* ***8.*** *Final dry and remove film by applying methanol*	***Scaffold***	S1	**0.5**	**1**	**0.06 ml**	**100 µl**	
		S2	**0.25**	**1**	**0.06 ml**	**100 µl**	
		S3	**0.05**	**1**	**0.06 ml**	**100 µl**	
		S4	**0.02**	**1**	**0.06 ml**	**100 µl**	
	Film	F1	**0.01**	**1**	**0.06 ml**	**100 µl**	
		F2	**0.005**	**1**	**0.06 ml**	**100 µl**	
		F3	**0.0025**	**1**	**0.06 ml**	**100 µl**	
		F4	**0.001**	**1**	**0.06 ml**	**100 µl**	

Table 2.10: Second Method

	Eggshell	Chitosan	Silk Fibroin	Glycerin
Scaffold	0.25 gr	1 ml	100 µl	0.125 ml
Film	0.001 gr	1 ml	100 µl	0.125 ml

2.2 Evaluation

The following measurements were obtaining for the physical properties of scaffolds and films.

2.2.1 Swelling of scaffold and film

Swelling behavior was examined in different buffer solution 1) PH:1.2, PBS solutions 2) PH: 7.4, PBS solutions; as shown in Figure 2.16. Size of scaffold and film were measured and divided by two each tested sample has 1 cm^2. Figure 2.17 and Figure 2.18 are shown the samples.

Figure 2.16: Swelling test

Figure 2.18: Film

Figure 2.17: Scaffold

The results are shown in Table 2.11 for the first method and Table 2.12 for the second method. Figure 2.19 and 2.20 are graphed for the following result and also Figure 2.21 and 2.22 are shown for second method results.

Table 2.11 Swelling Test First Method

Type I		**Swelling**								
Time (s)/ Weight		0	10	20	35	55	80	110	145	175
Scaffold **S2**	*PH=7.4*	**0.052**	**0.099**	**0.146**	**0.0135**	**0.011**	**0.11**	**0.083**	**0.073**	**0.045**
	PH=1.2	**0.068**	**0.106**	**0.124**	**0.134**	**0.128**	**0.114**	**0.106**	**0.083**	**0.058**
Film F4	*PH=7.4*	**0.0122**	**0.073**	**0.0462**	**0.033**	---	---	---	---	---
	PH=1.2	**0.022**	**0.16**	**0.113**	**0.11**	---	---	---	---	---

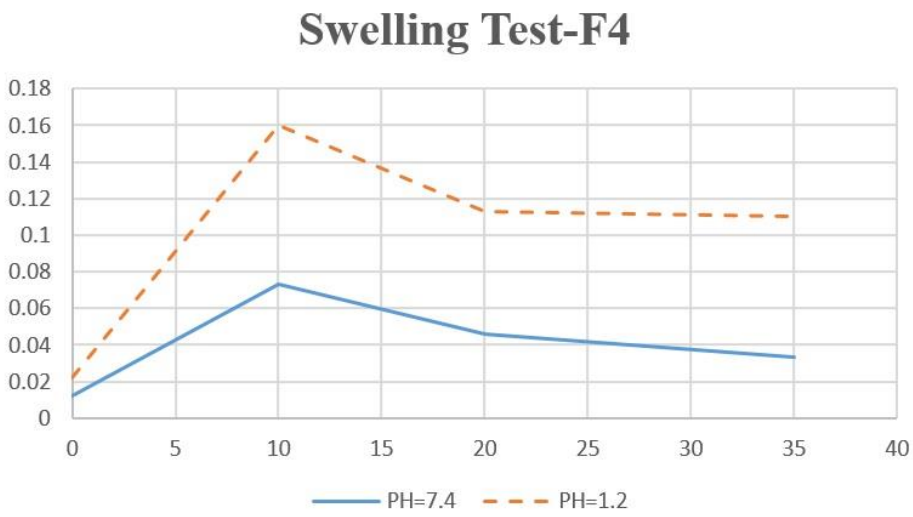


Figure 2.19: Swelling Test Graph Film 4 (gr/t(s))

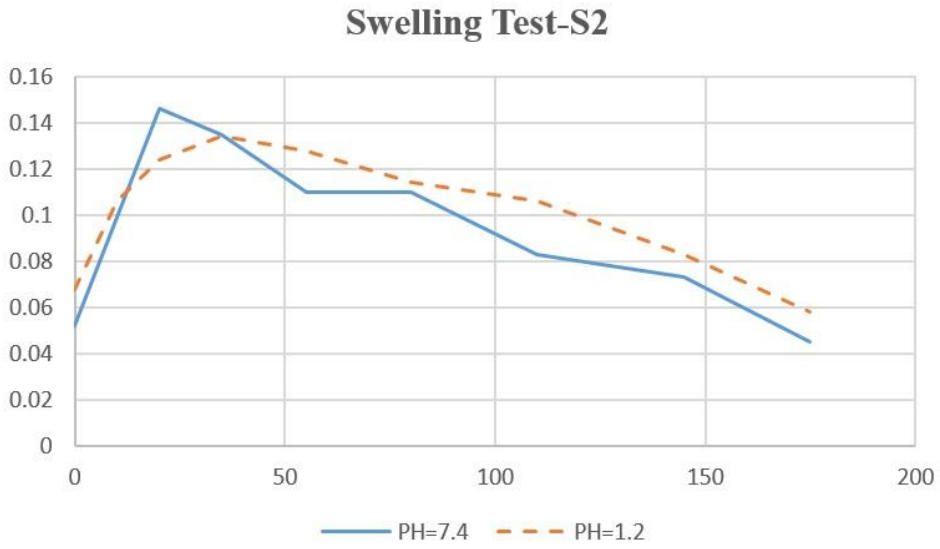


Figure 2.20: Swelling Test Graph Scaffold 2 (gr/t(s))

Table 2.12: Swelling Test Second Method

Type II		**Swelling**											
Time /Weight		0*(s)*	10*(s)*	20*(s)*	35*(s)*	55*(s)*	80*(s)*	110*(s)*	145*(s)*	175*(s)*	1hr	10hr	24 hr
Scaffold	*PH=7.4*	0.048	0.059	0.065	0.068	0.068	0.069	0.073	0.073	0.075	0.07	0.08	constant
	PH=1.2	0.049	0.07	0.086	0.105	0.12	0.128	0.46	0.151	0.17	0.15	0.039	dissolved
Film	*PH=7.4*	0.01	0.033	0.038	0.042	0.035	0.042	0.05	--	--	--	--	constant
	PH=1.2	0.005	0.016	0.02	0.023	0.028	0.038	0.097	--	--	--	--	dissolved

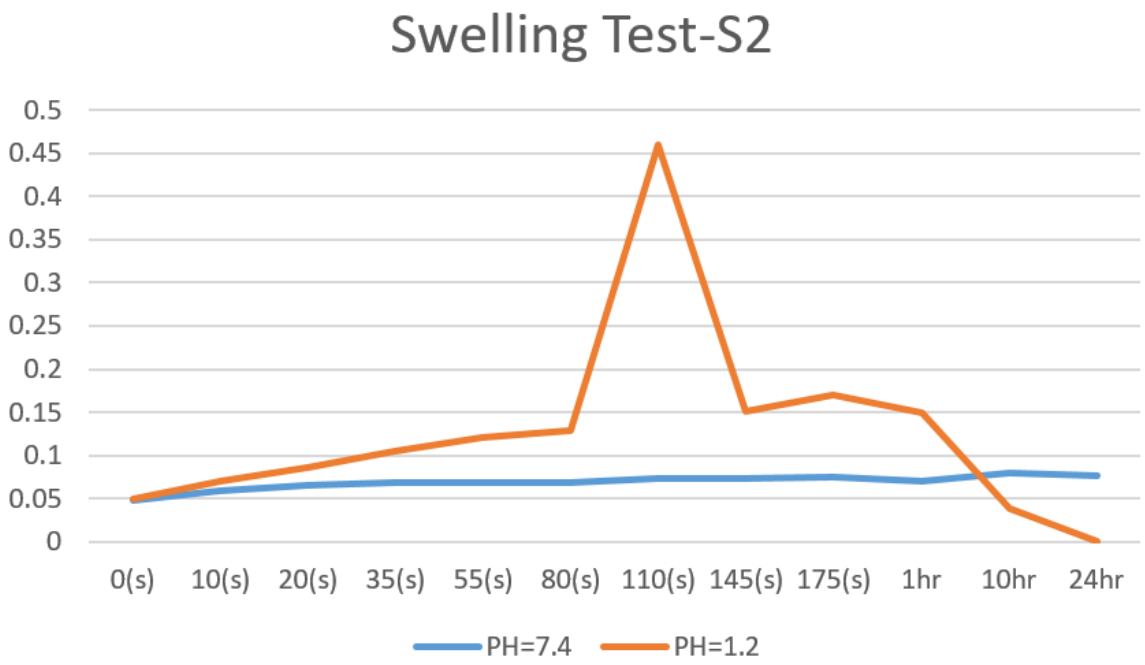


Figure 2.21: Swelling Test Graph Scaffold 2 (gr/t)

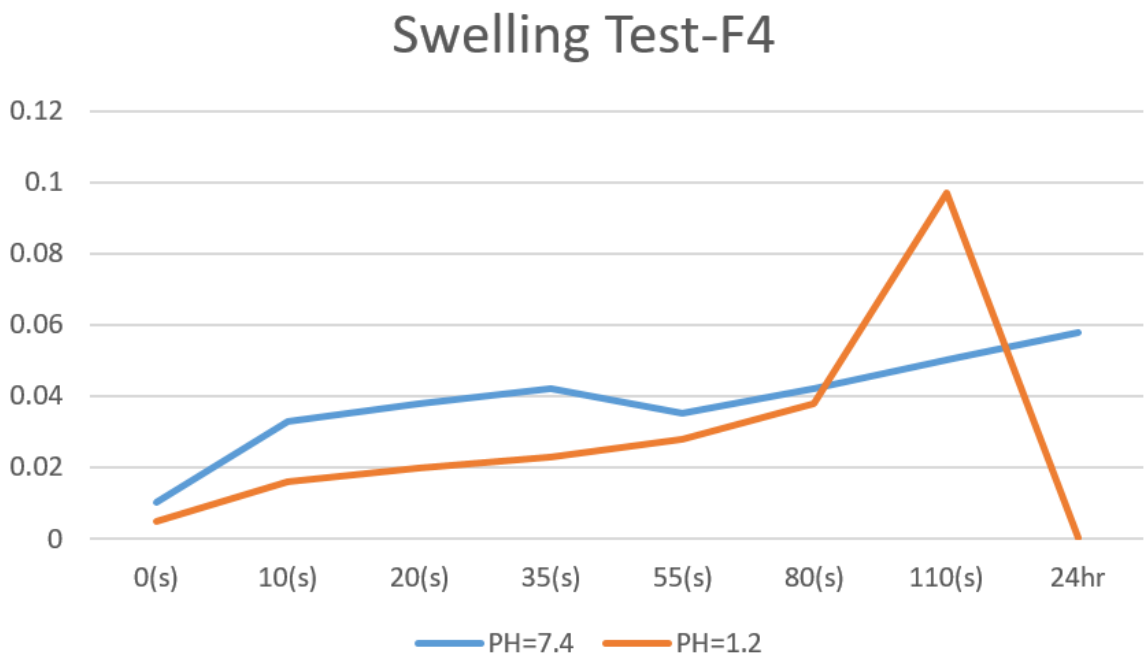


Figure 2.22: Swelling Test Graph film 4 (gr/t)

2.2.2 Moisture of scaffold and film

The moisture of the scaffold and film of method II was determined by drying the sample at 70 °C for 3 hours in an incubator and calculate the percentage of moisture content by equation 2.1.

$$\text{Moisture content (\%)} = \frac{w_1 - w_2}{w_1} \times 100 \qquad (2.1)$$

W_1 = Before drying (sample) gr

W_2 = After drying (sample) gr (SU-IL PARK et al., 2004)

$$\text{Moisture content Scaffold (\%)} = \frac{0.0613\ \text{gr} - 0.059\text{gr}}{0.0613\text{gr}} \times 100 = 3{,}75\ \%$$

$$\text{Moisture content Film (\%)} = \frac{0.0196\ \text{gr} - 0.0171\text{gr}}{0.0196\text{gr}} \times 100 = 12{,}75\ \%$$

2.2.3 Biodegradation of scaffold and film

Samples were tested by protease enzyme that was obtain from Feral N enteric draje tablet (Protease 400 FIP U). The first step was removing the cover of the tablet using a sharp knife; when the white peel was removed then the drug was grinded into powder. 0.01 gr of powder was dissolved into 25 ml pure water for scaffold and another exact amount of solution for film. Second solution was prepared by PBS and 0.01 gr powder for second dry scaffold and same exact volume and amount was made for second film. 2 samples from scaffold and 2 samples film (1 cm^2) were prepared and weighted; Secondly, two solutions were made. Pure water and PBS (same as Part 2.1.2 that was explained). Samples were placed into solution and kept for 72 hrs. finally the samples were dried and weighted. The degradation ratio for each samples were calculated by Equation 2.2 and the final result is shown in Table 2.13.

$$\text{Degradation Ratio} = \frac{\text{Initial dry weight} - \text{after degradation}}{\text{Initial dry weight}} \qquad (2.2)$$

During 72 hrs, they were kept at room temperature. The process is shown in Figure 2.23. (Prasong Srihanam, Wilaiwan Simchuer. 2009).

Table 2.13: Biodegradation

Solution	Sample	Initial Wight (gr)	After Degradation (gr)	Degradation ratio
Pure Water	**Film**	0.0085	0.0044	0.48
	Scaffold	0.033	0.0274	0.16
PBS	**Film**	0.004	0.0036	0.1
	Scaffold	0.036	0.038	-0.05

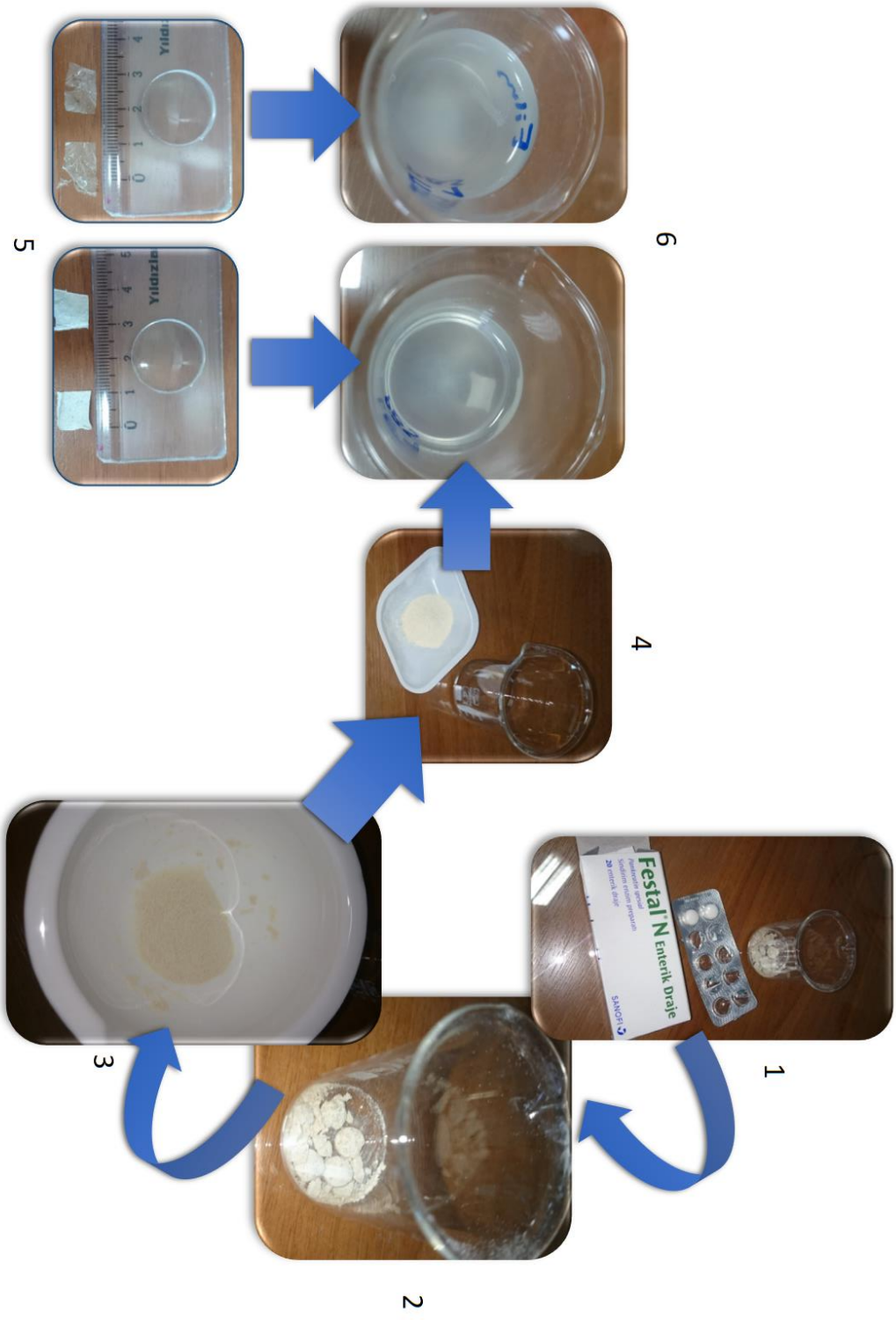


Figure 2.23: Biodegradation

2.2.4 Blood compatibility of scaffold and film

Fresh blood was taken from a healthy person was centrifuged for 5 min, 8000 RPM, the plasma was taken out from the tube shown in Figure 2.24. In the third step, plasma was added into the dishes where samples were kept then they put in an incubator at 37ºC for 5 min. For the next step, samples were washed completely with pure water and the Giemsa dye was added to the dishes and kept for about 8 min in an incubator, T=37 ºC. Lastly, they were washed and kept for 1 min in pure water T=37 ºC and were dried. Samples were analyzed under the microscope which is shown in Figure 2.25.

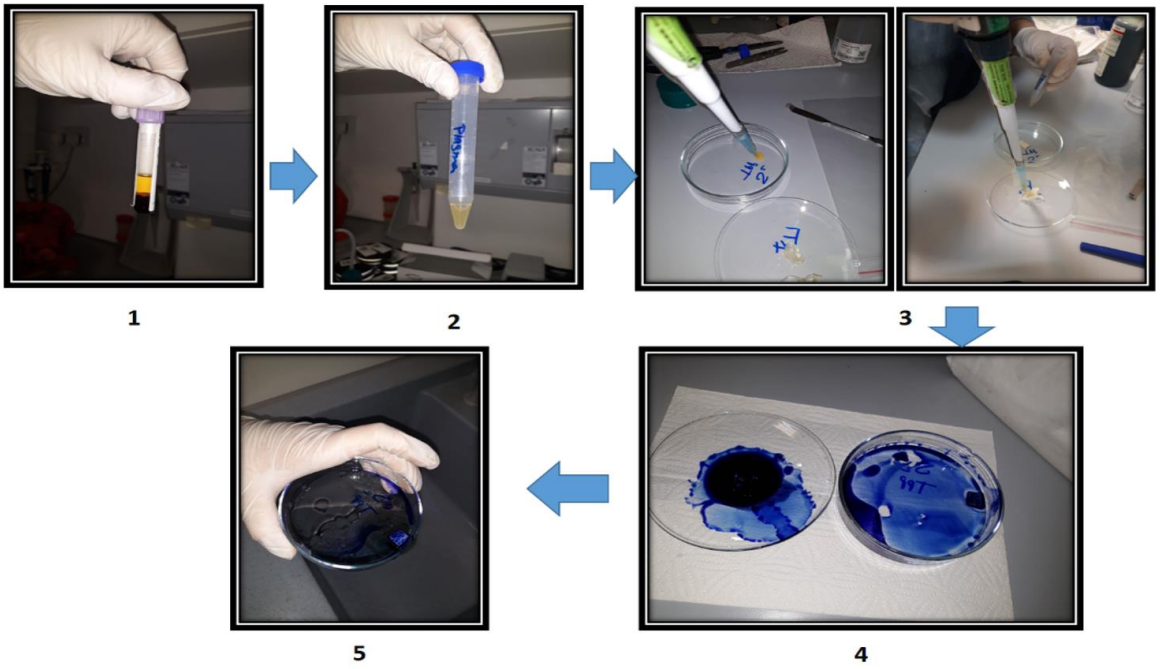
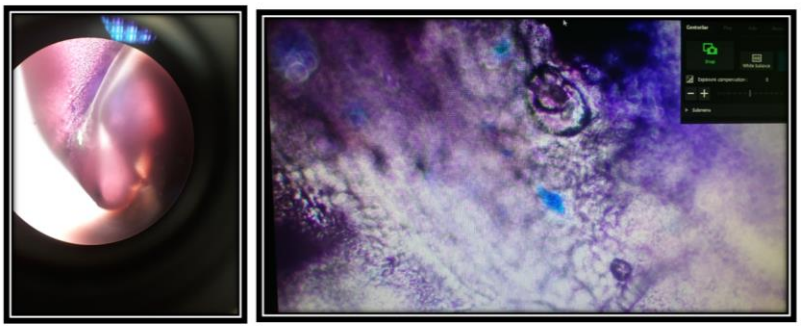


Figure 2.24: Platelet adhesion test. 1.Blood sample 2. Plasma 3. Covering the samples by plasma 4. Giemsa dye 5. Washing with water

Film



Scaffold

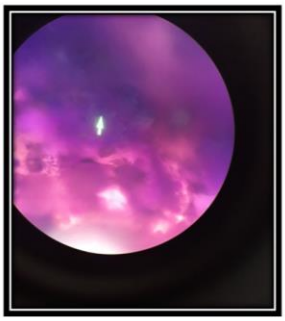


Figure 2.25: Film and Scaffold under Microscope

2.3 Fourier Transform Infrared (FTIR) Spectroscopic Analysis

Samples were tested by IR prestige-21 Fourier-Transform-Infrared-Spectrophotometer FTIR. The result showed as following from Figure 2.26 to Figure 2.30. The film graph is quite similar to Cs graph with a slight difference in wavelength and because of a lower amount of E.S and SF in its structure, there is not any visible peak in it. The minimum and maximum of the wavelength is between 83.30 - 101.61 µm. The amount of E.S in scaffold is more and it can be mentioned that there are some peaks that show $CaCO_3$ bond and because of bulk structure the wavelength cannot easily pass. The wavelength range is between 98.74 – 101.61 µm. With the help of Table 2.14 till Table 2.17, we can find the organic bond in the graph.

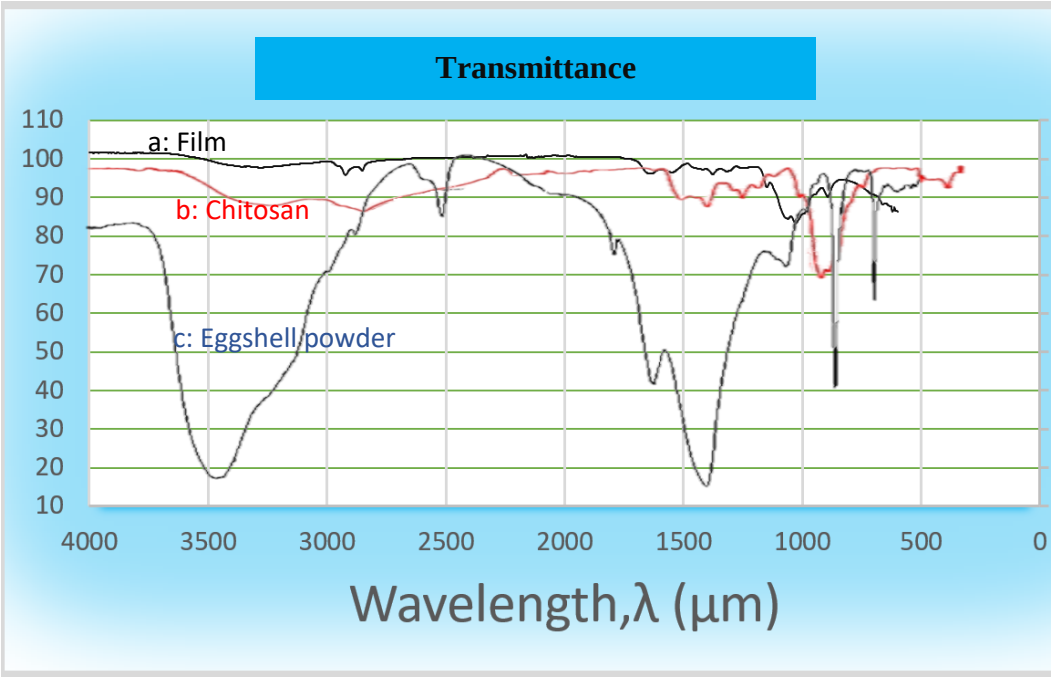


Figure 2.26: FTIR analysis for Film- native Cs (Esquivel Reynaldo et al. 2015)-ES (Rajan Choudhary et al. 2015)

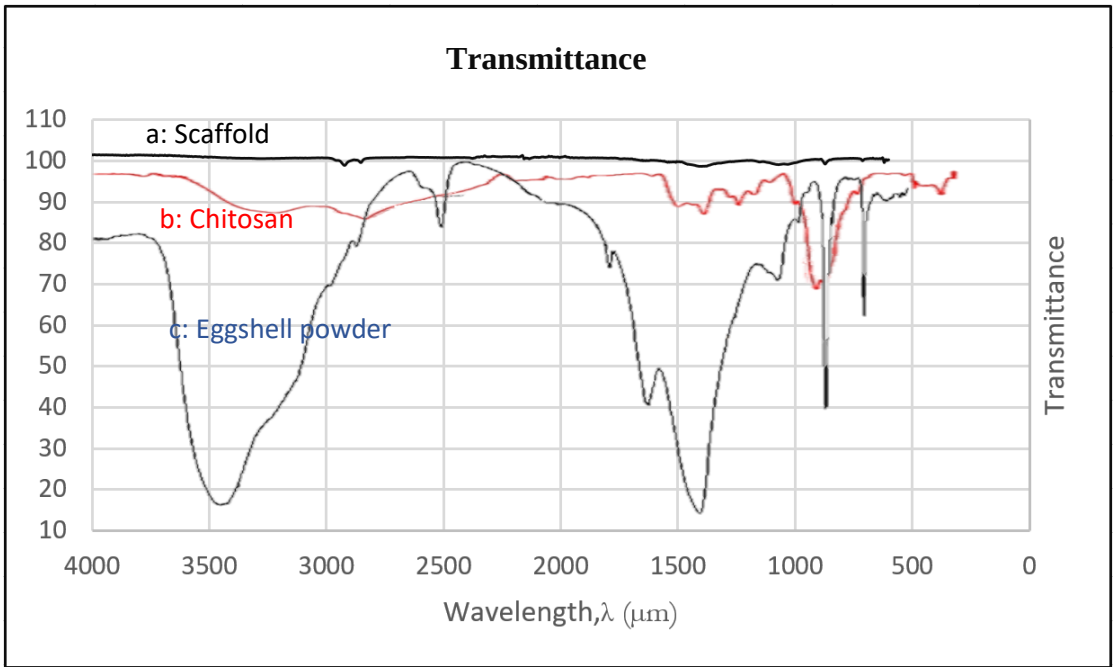


Figure 2.27: FTIR analysis for Scaffold- native Cs (Esquivel Reynaldo et al. 2015)-ES (Rajan Choudhary et al. 2015)

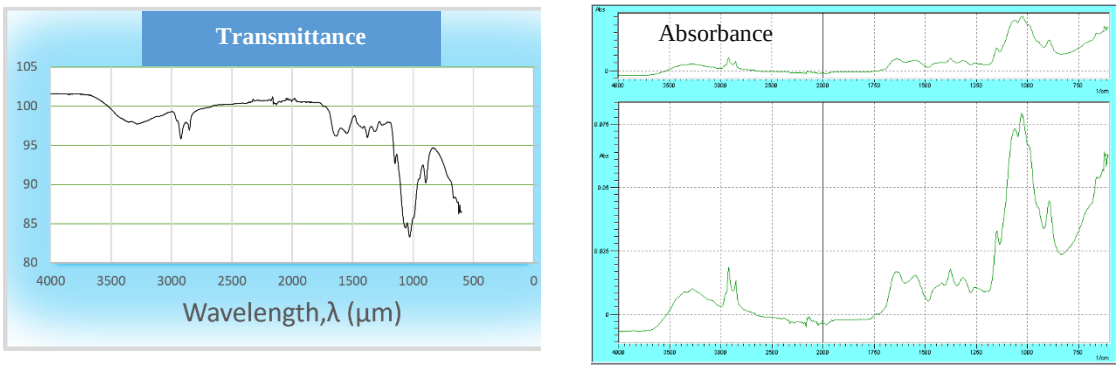


Figure2.24: FTIR analysis for Film transmittance and absorbance

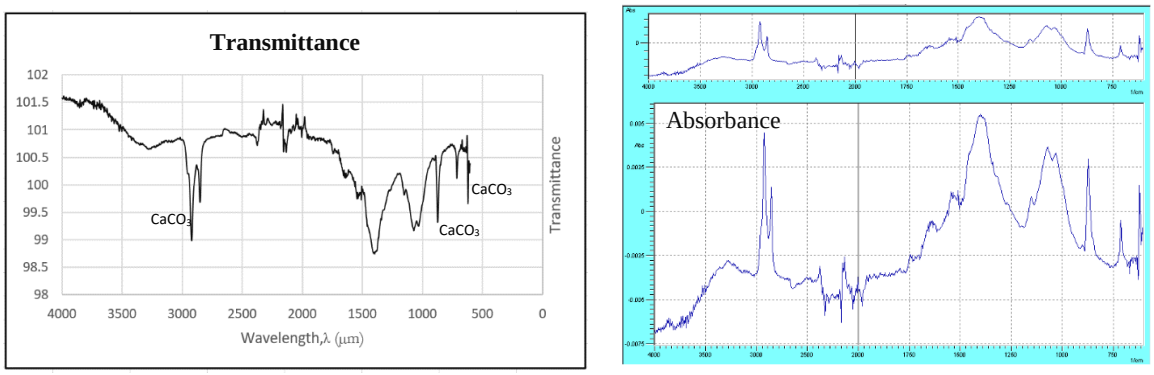


Figure 2.29: FTIR analysis for Scaffold transmittance and absorbance

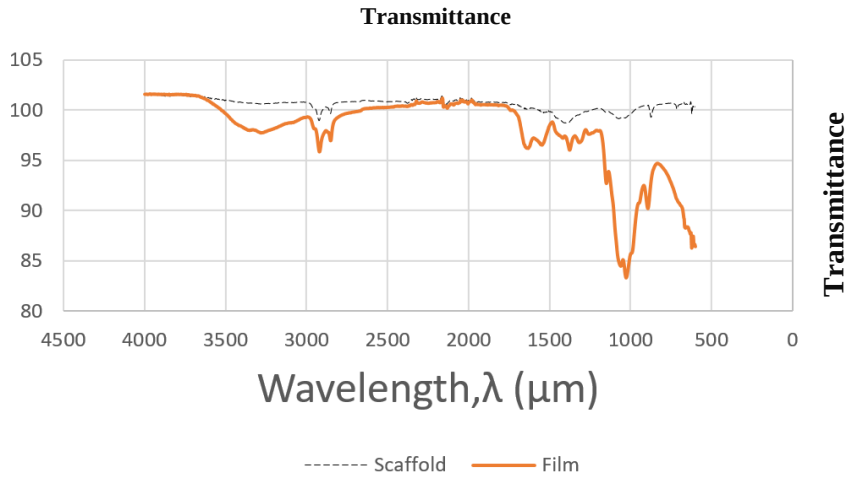


Figure 2.30: FTIR analysis for Scaffold & Film

Table 2.14: Characteristic FTIR of Organic Functional Groups (1) (Silverstein, et al. 1981)

Analysis of C-H out-of-plane bending can often distinguish substitution patterns			
Carbonyl			
C=O	stretch	1670-1820	strong
(conjugation moves absorptions to lower wave numbers)			
Ether			
C-O	stretch	1000-1300 (1070-1150)	strong
Nitrile			
CN	stretch	2210-2260	medium
Nitro			
N-O	stretch	1515-1560 & 1345-1385	strong, two bands

Table 2.15: Characteristic FTIR of Organic Functional Groups (2) (Silverstein, et al. 1981)

Functional Group	Absorption (cm^{-1})	Intensity	Comments
Alkane			
C-H	2850 - 2960	Medium	Sharp
Alkene			
=C-H	3020 - 3100	Medium	
C=C	1640 - 1680	Medium	
Alkyne			
≡C-H	3300	Strong	
C≡C	2100 - 2260	Medium	
Alkyl halide			
C-Cl	600 - 800	Strong	
C-Br	500 - 600	Strong	
Alcohol			
O-H	3400 - 3650	Strong	Often broad
C-O	1050 - 1150	Strong	
Arene			
C-H	3030	Weak	
C=C	1660 - 2000	Weak	
C=C	1450 - 1600	Weak	

Functional Group	Absorption (cm^{-1})	Intensity	Comments
Amine			
N-H	3300 - 3500	Medium	Often broad
C-N	1030 - 1230	Medium	
Aldehyde and ketone			
C=O	1670 - 1800	Strong	
Carboxylic acid			
C=O	1660 - 1740	Strong	
O-H	2500 - 3200	Strong	Often broad
Ester			
C=O	1670 - 1750	Strong	
Amide			
C=O	1630 - 1690		
N-H	3140 - 3500	Strong	1°: two bands 2°: one band
Nitrile			
C≡N	2210 - 2260	Medium	
Nitro			
NO_2	1540	Strong	

Table 2.16: Characteristic FTIR of Organic Functional Groups (3) (Silverstein, et al. 1981)

IR Absorption Frequencies of Functional Groups Containing a Carbonyl (C=O)			
Functional Group	Type of Vibration	Characteristic Absorptions (cm-1)	Intensity
Carbonyl			
C=O	stretch	1670-1820	strong
(conjugation moves absorptions to lower wave numbers)			
Acid			
C=O	stretch	1700-1725	strong
O-H	stretch	2500-3300	strong, very broad
C-O	stretch	1210-1320	strong
Aldehyde			
C=O	stretch	1740-1720	strong
=C-H	stretch	2820-2850 & 2720-2750	medium, two peaks
Amide			
C=O	stretch	1640-1690	strong
N-H	stretch	3100-3500	unsubstituted have two bands
N-H	bending	1550-1640	
Anhydride			
C=O	stretch	1800-1830 & 1740-1775	two bands
Ester			
C=O	stretch	1735-1750	strong
C-O	stretch	1000-1300	two bands or more
Ketone			
acyclic	stretch	1705-1725	strong
cyclic	stretch	3-membered - 1850	strong
		4-membered - 1780	
		5-membered - 1745	
		6-membered - 1715	
		7-membered - 1705	
a,b-unsaturated	stretch	1665-1685	strong
aryl ketone	stretch	1680-1700	strong

Table 2.17: Characteristic FTIR of Organic Functional Groups (4) (Silverstein, et al. 1981)

Characteristic IR Absorption Frequencies of Organic Functional Groups			
Functional Group	Type of Vibration	Characteristic Absorptions (cm-1)	Intensity
Alcohol			
O-H	(stretch, H-bonded)	3200-3600	strong, broad
O-H	(stretch, free)	3500-3700	strong, sharp
C-O	(stretch)	1050-1150	strong
Alkane			
C-H	stretch	2850-3000	strong
-C-H	bending	1350-1480	variable
Alkene			
=C-H	stretch	3010-3100	medium
=C-H	bending	675-1000	strong
C=C	stretch	1620-1680	variable
Alkyl Halide			
C-F	stretch	1000-1400	strong
C-Cl	stretch	600-800	strong
C-Br	stretch	500-600	strong
C-I	stretch	500	strong
Alkyne			
C-H	stretch	3300	strong,sharp
–C≡C–	stretch	2100-2260	variable, not present in symmetrical alkynes
Amine			
N-H	stretch	3300-3500	medium (primary amines have two bands; secondary have one band, often very weak)
C-N	stretch	1080-1360	medium-weak
N-H	bending	1600	medium
Aromatic			
C-H	stretch	3000-3100	medium
C=C	stretch	1400-1600	medium-weak, multiple bands

CHAPTER 3

RESULT AND DISCUSSION

3.1 Result and Discussion

The biomaterial that is used for tissue regeneration must be mimicking the ECM properties, some biomaterials will not be suitable because synthesizing and combining of them, produce new properties that chemically and physically are harmful to the cell. Nowadays biomaterials are made of natural polymers are more considered. As mentioned before the aim of this study was the preparation of scaffold and film from green waste for specifically bone regeneration.

Two methods that are mentioned in this research are fabricated from the same material but different amounts and the technique of preparation.

Samples were prepared, then from physical appearance and uniform concentration of composites, one from the scaffold and one from the film were chosen for analyzing. Scaffold and film that contained 0.25 gr and 0.001 gr ES respectively from each method were selected.

The first method Cs solution had the high concentration of AA. The acidic environment made the sample be less promising for cell culture, and also in the first step of swelling test, it was shown scaffold and film degraded within seconds and dissolved completely in solution in acidic and basic. So that the other tests were not applied on samples from the first method.

The second method got the better result in swelling test. The results were acceptable and showed that in PH=7.4 samples had more tolerance within 15 days' observation. Compare to acidic solution scaffold and film were degraded slowly after 24 hours. Cs in the acidic environment or PH=2 has better swelling behavior so that it will use in drug delivery application rather than scaffold, and it is referring to the ionization of degree in the amino group. The consequence of this observation was indicated both samples are the good candidate for the biological system that has PH= 7.4.

The percentage of moisture was obtained 3.75 % for scaffold and 12.75 % for biofilm in 70℃ during 3 hrs that as a result shows biofilm will lose water 4 times more than the scaffold.

Biodegradation by protease enzyme was tested. The results showed that the protease is able to degrade the samples. Degradation indicated better result in pure water rather than PBS (PH=7.4). In PBS solution, the scaffold was slight swelling and film was degraded only 10% of its mass. Comparing to water solution; scaffold and film were degraded 17% and 48% respectively and physically the structure of both samples were denatured.

Peripheral Streaming method for platelet adhesion analysis was tested and the results showed both samples (scaffold and film) are quite biocompatible due to the dye region.

The FTIR graph was performed and shown the difference between the scaffold and film which wave could pass through the film but due to the structure of scaffold, the wave could not pass easily. The similarity to native Cs graph showed the bonding structure of the film was quite similar. Presenting of $CaCO_3$ bond in some peaks in scaffold was obvious but the Transmittance range was slightly different from the native E.S powder. As a result, it shows the similarity of native Cs and ES but the range was more closed in both sample.

Although they are lots of researchers that worked on Cs, SF, and ES separately or combination of two, none of them fabricate scaffold and film from all these three combinations. The majority of studies were based on freeze-drying technique and they have claimed that chitosan and silk fibroin is suitable for the living system, due to their mechanical properties, water, and O_2 permeability. SF and ES was another common biomaterial that was used in the scaffold, extracted HA from ES for bone formation. Availability and fast healing in bone were two main reasons for using ES in the scaffold. The high number of researchers were work on ESM rather than ES and the reason was the ability to dissolve in acidic solution and soluble protein was used in the film as bioactive.

It is promising that this research is the new way to manufacture scaffold and film from the natural waste material for bone regeneration which needs the long-term study with the different characteristic test.

CHAPTER 4

CONCLUSION

4.1 Conclusion

The new way of using waste biomaterial in the application, due to their natural and similar properties to the biological system was the main idea of this research. Cs/SF/ES scaffold and biofilm are promising candidates in tissue engineering specifically for bone and cartilage regeneration. Their tolerance in acid and basic environment, biocompatible, non-toxic and their viscosity make them be the suitable host for the living system. Gelation properties of Cs build flexible scaffold/biofilm so that external and internal forces do not make the network break or degrade easily. Cell adhesion due to polysaccharide group is similar to GAGs in ESM. One significant effect of surface treatment on both samples is to wash surface completely with pure water after treating, otherwise, it will block the surface pores.

Designing scaffold based on different tissue requires specific biomaterial by using suitable surface modification. Comparing between scaffold and film; the performance in mechanical tolerance due to stress, the scaffold was the better candidate but estimation of cell culture, the film would be the best candidate due to its flexibility and transparent planar formation. One disadvantage of the 3-D scaffold was difficult to observe the pore structure because light could not pass through it so that the result would not be measurable easily.

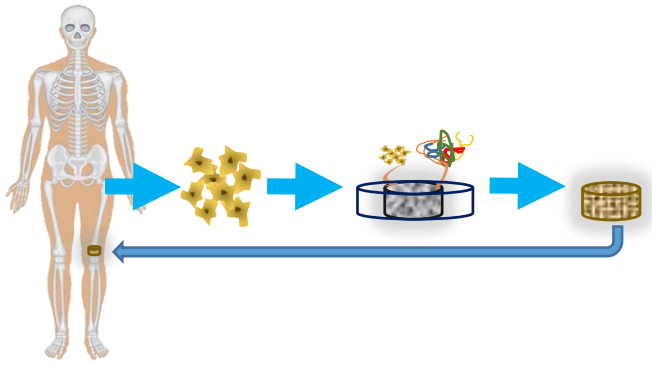


Figure 4.31: Simulation

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