REPUBLIC OF TURKEY
YILDIZ TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

PROTEIN-BASED BIOCOMPOSITE CRYOGELS AS TISSUE ENGINEERING SCAFFOLDS

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MSc. THESIS
DEPARTMENT OF BIOENGINEERING
PROGRAM OF BIOENGINEERING

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İSTANBUL, 2016

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A thesis submitted by Kübra Burcu AKKAYA in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** was approved by the committee on the 01.04.2016 in the Department of Bioengineering, Bioengineering Program.

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This study was supported by the Scientific Research Project Coordinator of Yıldız Technical University Grant No: 2014-07-04-YL03.

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor Assist. Prof. Mehmet Murat Özmen, for his valuable advice and guidance from the beginning to the end of this study. I would also like to acknowledge Assist. Prof. Jennifer Patterson for her support and guidance during cell studies part of the study. I also would like to thank all of the members of Yıldız Technical University (YTU), Bioengineering Department and KU Leuven Materials Engineering Department (MTM) for their endless help.

I would like to thank my helpful and wonderful lab-mates and friends at both YTU and MTM, especially, Dr. Agnieszka Franczak, Laurien Van den Broeck, Annabel Braem, Sally Louis Homsy, Abhijith Kundadka Kudva, Kimon Alexandros Leonidakis, Maria Montero, Ehsan Shafaian, Maarten Van Den Plas, Boyu Chen, Begüm Aytuğrul, Tuğçe Şen, Pieter L'hoëst and Britt Vandenbroeck for their help and support during the study.

A big thank to Prof. Bülent Topbaşı, a member of Marmara University, Faculty of Dentistry, for his fruitful advice and support during my academic life. I also would like to thank my dear friends, Esra Aydın, Saliha Özdemir, Merve Reyhan Kayıkçı, Meryem Çoban, Zeynep Büşra Bolat, Kübra Ünlü, Melek Karaca and Hüsna Karaboğa for their encouragement and help.

Finally, my deepest thanks go to my lovely family – especially to my mother Müzeyyen Akkaya, my father Fehmi Akkaya, my sister Kadriye Arzu Ceylan and her spouse Aşkın Ceylan and their children, my niece Rahile Sude Ceylan and my nephew Yusuf Kerem Ceylan, for their endless love and support throughout my entire life.

I believe that everything has a reason in our lives. Every person and every event teach us something. I would like to end with thanking everyone who helped me to grow up and become a better person.

March, 2016

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LIST OF SYMBOLS

^{o}C	The degree Celcius
μm	Micrometers
C_0	Initial polymer concentration
Ca-P	Calcium-Phosphate
cells/ml	The number of cells per milliliters
CO_2	Carbondioxyde
D_0	Diameter of the gel after swelling
D	Diameter of the gel after preparation
Da	Dalton
D_{dry}	Diameter of the dry gels
g	grams
g/ml	Density
g/mol	Molar mass
kDa	Kilodalton
M	Molar Concentration
M_{dry}	Mass of the dry gels
ml	Mililiters
m_{rel}	Relative mass parameter
mm	Milimeters
m_t	Mass of the gel sample at time *t*
nm	Nanometers
OH^-	Hydroxyl group
pH	Power of Hydrogen
q_v	Equilibrium volume swelling ratio
q_w	Equilibrium weight swelling ratio
sec	Seconds
t	Times in seconds
V_0	Volume of gel after preparation
V	Volume of swollen gel
V_{dry}	Volume of the dry gel
V_{eq}	Equilibrium volume swelling ratio
W_g	The gel fraction

LIST OF ABBREVIATIONS

10T1/2	Mouse embryonic fibroblast cell line
Arg-Gly-Asp	Arginine-Glycine-Aspartic Acid
BMPs	Bone morphogenic proteins
C-MP	Cryogel / Microparticle Composite
C2C12	Mouse myoblasts cell line
Cos-7	African green monkey fibroblast cell line
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDC-NHS	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide-N-hydroxysuccinimide
FBS	Fetal Bovine Serum
FTIR	Fourier transform infrared spectroscopy
GCH	Gelatin/chondoitin-6-sulfate/hyaluronan
HA	Hydroxyapatite
HeLa	Human cervical cancer cells
HEMA	Hydroxyethylmethacrylate
IMR-32	Human neuroblastoma cell line
L929	Mouse fibroblast cell line
MC3T3-E1	Mouse preosteoblastic cell line
MG-63	Osteoblast-like osteosarcoma cell line
MSCs	Mesenchymal stem cells
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NCI-H460	Human lung cancer cells
NIH 3T3	Mouse embryonic fibroblast cell line
OVA	Ovalbumin
OVA/HA	Ovalbumin/Hydroxyapatite
PBS	Phosphate Buffered Saline
PEG	Polyethylene glycol
PGA	Polyglycolic acid
PHEMA	Poly(2-hydroxyethyl methacrylate)
PLA	Polylactic acid
PLGA	Poly(lactic-co-glycolic acid)
PTAC	PVA–tetraethylorthosilicate-alginate-calcium oxide
PVA	Polyvinyl alcohol
RGD	Arginylglycylaspartic acid
RNA	Ribonucleic acid

SAOS-2	Human osteosarcoma cell line
SBF	Stimulated body fluid
SEM	Scanning Electron Microscopy
Tgase	Transglutaminase
TE	Tissue Engineering
XRD	X-ray Diffraction
FTIR	Fourier transform infrared spectroscopy

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ABSTRACT

PROTEIN-BASED BIOCOMPOSITE CRYOGELS AS TISSUE ENGINEERING SCAFFOLDS

Kübra Burcu AKKAYA

Department of Bioengineering

MSc. Thesis

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The aim of this thesis is to prepare various protein-based macroporous hydrogels (cryogels) and to investigate their potential as scaffolds for bone tissue engineering applications.

This study is unique to explore cell viability and biocompatibility of ovalbumin/hydroxyapatite cryogel scaffolds as tissue engineering constructs. Here, ovalbumin cryogels were prepared in aqueous solutions by cryogelation method in which the crosslinking reactions are carried out at temperatures below the freezing point of the reaction solvent. It was decided to prepare ovalbumin cryogels by non-covalent interactions in order to eliminate the harmful effects of the toxic chemical crosslinkers such as glutaraldehyde on the cells. Ovalbumin was dissolved in aqueous urea solutions, and then these solutions were kept at subzero temperatures. This process resulted in unfolding of ovalbumin protein and formed cryogels via hydrophobic and electrostatic interactions. Using this method, novel ovalbumin/hydroxyapatite cryogels were obtained with superior porous structure and mechanical properties. The influence of varying polymer concentration and hydroxyapatite amount on the cryogel properties were investigated.

All of the produced cryogels were characterized by gel fraction, equilibrium swelling, swelling kinetics, mechanical properties and internal morphology measurements. The porosity of the cryogels was observed by scanning electron microscopy (SEM). The results revealed that, the produced cryogels have interconnected macroporous structure with pore size of 30-70 μm. The swelling results of the gels showed that the dry samples could swell 10-30 folds of their drying weights in a few seconds. Furhermore, the cryogel could be compressed totally without any crack formation. These results proved that the cryogels could be used as scaffolds for tissue engineering applications.

In the last part of the study, ovalbumin/hydroxyapatite biocomposite cryogels were combined with 10T1/2 mouse embryonic fibroblast cells *in vitro*. Biocompatibility of cryogel scaffolds was investigated by using these fibroblast cells. The viability and proliferation of 10T1/2 fibroblast cells that is the biocompatibility of the cryogels, was explored within these scaffolds. The results of the *in vitro* cell studies showed that the ovalbumin/hydroxyapatite biocomposite cryogels were preferable biomaterials than ovalbumin cryogels as they provide a better environment for the cells.

Keywords: Ovalbumin, hydroxyapatite, cryogel, composite scaffold, tissue engineering.

ÖZET

DOKU MÜHENDİSLİĞİ DOKU İSKELESİ OLARAK PROTEİN BAZLI BİYOKOMPOZİT KRİYOJELLER

Kübra Burcu AKKAYA

Biyomühendislik Bölümü

Yüksek Lisans Tezi

Danışman: Mehmet Murat ÖZMEN

Bu tezin amacı protein bazlı makrogözenekli hidrojellerin (kriyojeller) hazırlanması ve doku iskelesi olarak doku mühendisliği için potansiyel uygulamalarının araştırılmasıdır.

Bu çalışmada ilk kez ovalbumin/hidroksiapatit doku mühendisliği iskeleleri hazırlandı ve bu iskelelerin karakterizasyonları yapılarak, hücrelerin canlılığı ve malzemelerin biyouyumluluğu incelendi. İlk olarak, ovalbumin kriyojelleri, çapraz bağlanma reaksiyonlarının çözücünün donma noktasının altındaki sıcaklıklarda gerçekleştiği kriyojelasyon yöntemi ile sulu ortamda hazırlandı. Kriyojellerin hazırlanmasında kullanılan gluteraldehit gibi çapraz bağlayıcılar hücreler üzerinde toksik etki gösterdiğinden, ovalbumin kriyojelleri kovalent olmayan etkileşimler ile sentezlendi. Ovalbumin, sulu üre çözeltilerinde çözülerek düşük sıcaklıklarda donduruldu. Bu işlem ovalbumin proteininin katlanmasına neden olarak hidrofobik/elektrostatik etkileşimler ile kriyojellerin sentezlenmesine olanak sağladı. Bu metod ile, üstün gözeneklilik ve mekanik özelliklere sahip yeni ovalbumin/hidroksiapatit kriyojelleri sentezlendi. Polimer konsantrasyonu ve hidroksiapatit miktarı gibi sentez parametrelerinin kriyojeller üzerindeki etkileri incelendi.

Üretilen bütün kriyojellerin jel fraksiyonu, denge şişme oranı, şişme kinetiği, mekanik özellikleri ve içyapı morfolojileri araştırıldı. Kriyojellerin gözenek yapıları taramalı elektron mikroskobu (SEM) ile incelendi. SEM ölçümü sonuçlarıyla, kriyojellerin 30-70 µm boyutlarında birbirleriyle bağlantılı gözenekli bir içyapıya sahip oldukları görüldü. Şime sonuçları ise kriyojellerin kuru ağırlıklarının 10-30 katı kadar suyu birkaç saniye içinde absorplayabildiklerini gösterdi. Ayrıca, kriyojellerin kuvvet altında, hiçbir çatlak oluşmadan tamamen sıkıştırılabildikleri görüldü. Bu sonuçlarla kriyojellerin doku mühendisliğinde potansiyel uygulamaları olan yeni biyomateryaller oldukları anlaşıldı.

Çalışmanın son kısmında ise, ovalbumin/hidroksiapatit biyokompozit kriyojelleri, *in vitro* ortamda 10T1/2 fare embriyonik fibroblast hücre hattı ile birleştirildi. Kriyojel doku iskelelerinin biyouyumlulukları bu fibroblast hücreleri kullanılarak araştırıldı. Bu bağlamda, 10T1/2 fibroblast hücrelerinin canlılığı ve yayılması diğer bir değişle; kriyojellerin biyouyumlulukları incelendi. Yapılan bu hücre kültürü çalışmaları, ovalbumin/hidroksiapatit biyokompozit kriyojellerinin ovalbumin kriyojellerine kıyasla hücrelerin yayılması ve çoğalması için daha uygun bir ortam oluşturduğunu gösterdi.

Anahtar Kelimeler: Ovalbumin, hidroksiapatit, kriyojel, kompozit doku iskelesi, doku mühendisliği.

CHAPTER 1

INTRODUCTION

1.1 Literature Review

1.1.1 Bone

Bone is mineralized connective tissue which is living, highly vascularized, dynamic and forms the skeleton structure of many vertebrates [1], [2]. Bone can be characterized by its hardness, strength and stiffness. Also, it maintains structural and mechanical support in order to protect the human body and vital organs [3]. Moreover, it supplies free hanging spaces for tendons and muscles and plays an important role in calcium homeostasis by being a crucial reservoir for vital minerals such as phosphate, magnesium and potassium. In addition to this, bone is involved in the production process of blood and stem cells and a balancing mechanism of bicarbonate ions as a result of adjusting the pH level of the body [4], [5].

In general, bone consists of organic and inorganic phases. The organic phase of bone is formed by collagen fibers (90%) and non-collagenous organic proteins which are condensed in the main matrix of bone. The main structural unit of collagen consists of three right-handed polypeptide fibrils which are 300 nm in length and 1.5 nm in diameter. This characteristic structure of collagen is called a triple helix form. Collagen is found in an organized form and regulates the calcification process into the bone microstructure. On the other hand, there are many proteins in addition to collagen such as growth factors, cytokines, osteonectin, osteocalcin, hyaluronan, proteoglycans, phospholipids and phosphoproteins which play a crucial role in bone regeneration and osteogenesis [6-9].

As mentioned earlier, bone has an inorganic phase that consists of minerals. This mineral substance is called hydroxyapatite which is a member of the calcium phosphate

family. Its molecular formula is $Ca_{10}(PO_4)_6(OH)_2$ and it has been thought that hydroxyapatite crystals fill the gaps between collagen fibers in bone structure. Hydroxyapatite is an important molecule for defining bone type because it deposits in the soft organic bone matrix and defines the mechanical characteristics of bone [10-12].

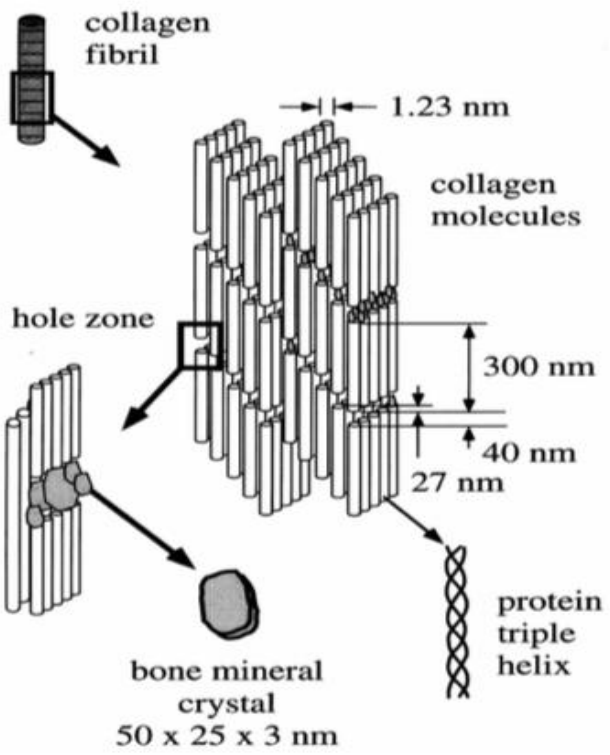


Figure 1. 1 The organisation of collagen fibers and hydroxyapatite crystals in bone microstructure [13]

As human beings, our skeleton system is composed of 80% cortical bone and 20% trabecular or cancellous bone. Cortical bone has a very dense and hard structure. Also, it contains many microscopic vessels [6], [13]. Cortical bone acts as a shield on the outer side of bone which supports and protects bone against physical external pressure. On the other hand, cortical bone has only 10% porosity. Therefore, it contains very limited space for cells and blood vessels. It is generally found in long bones [3], [15].

Trabecular bone presents a spongy-like structure, which consists of 50-90% pores and has an interconnected network of trabecula (small bone trusses). Trabecular bone contains bone marrow and blood vessels so it has low mechanical strength compared to cortical bone. It is found at the end of long bones, fetus and vertebra [3], [15].

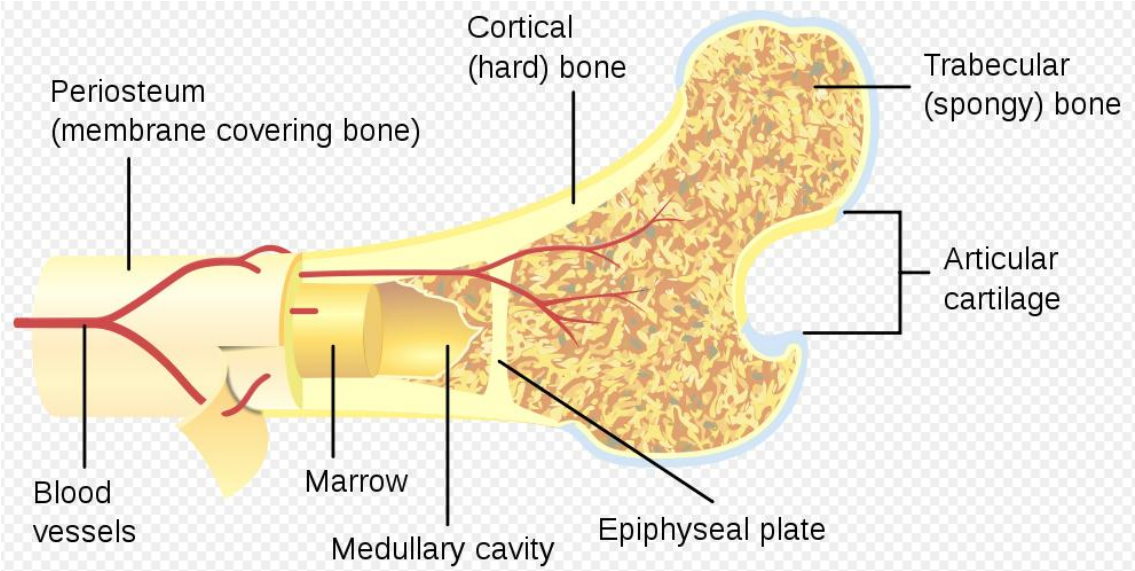


Figure 1. 2 Cortical and Cancellous Bone [1]

Being a living tissue, bone can rebuild itself with the assistance of special cell types like osteoblasts, osteocytes and osteoclasts [3].

Osteoblasts are mononuclear cells which are responsible for the production of bone and are found on the surface of bone [3]. Moreover, they are responsible for the production of organic collagenous proteins in the bone matrix [15]. On the other hand, osteoblasts play a role in the generation of some hormones like prostaglandins. Such hormones affect bone mineralization with the aid of alkaline phosphatase which is an enzyme produced by prostaglandins. When a calcified matrix covers osteoblasts, they turn into osteocytes [15].

Osteocytes, dendritic shaped mature osteoblasts, are embedded into bone matrix [3] and comprise approximately 90-95% of total bone cells [16]. Moreover, osteocytes are characterized by being responsive cells in the conversion of mechanical stimuli into biomolecular molecules, which activate proper bone formation [17].

The third cell type is osteoclast, which is specialized for bone resorption [17], [19]. These large, multinucleated cells have well-defined and developed organelles. Moreover, unlike osteoblasts and osteocytes, osteoclasts are reproduced from hematopoietic cell lines of macrophage/monocyte lineage [20].

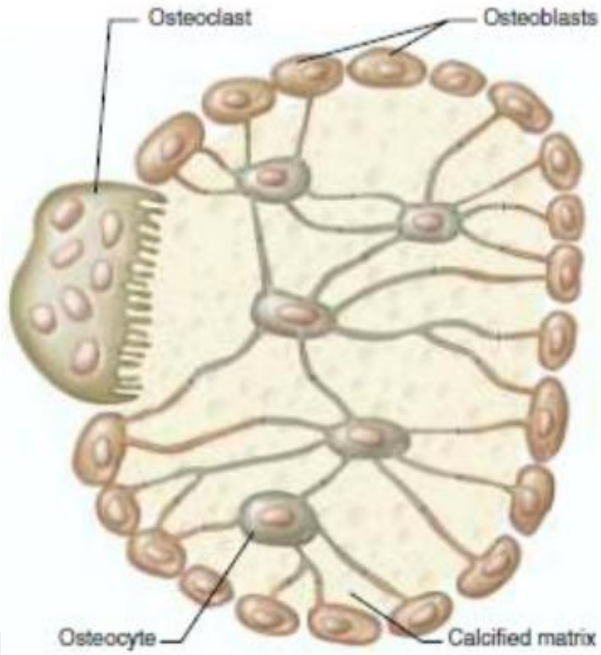


Figure 1. 3 A scheme of a portion of small bone. Osteocytes seem like dendritic cells and osteoblasts make tight junctions [18]

1.1.2 Healing of Bone

When an injury has occurred, several processes are initiated immediately to heal the defected part of the bone and many differentiating and growth factors are activated [21]. First of all, pluripotent osteoprogenitor cells which are known as bone morphogenic proteins (BMPs) and bound to collagen very closely. These osteoconductive proteins accompanied by other growth factors, cytokines, and hormones work to activate migration and differentiation of mesenchymal cells. As in the other tissues, the bone repairing process has continuous and dynamic cascades [21]. These cascades are shown in Figure 1.4.

The healing process of bone is initiated with a rapid inflammatory response, which takes minutes to hours followed by chemotaxis, and mitosis which can occur in hours to days. The final processes complete bone healing as the new extracellular matrix is produced, the defected site is remodeled and local angiogenesis is initiated which can take days to weeks to complete [21].

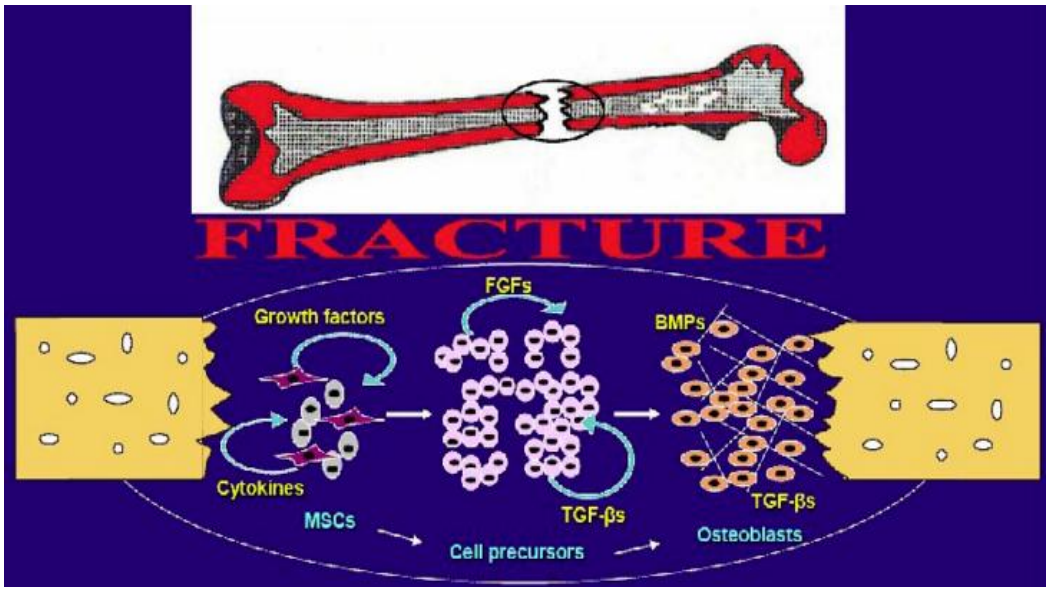


Figure 1. 4 Growth factors and cytokine production during bone regeneration, remodeling and repair process [22]

1.1.3 Current and Modern Treatments of Bone Defects

Autograft transplants are considered as the gold standard for bone repair methods because they call osteogenic, osteoinductive and osteoconductive components to the defect sites without any host immune response reactions [23]. On the other hand, an autograft transplantation process requires several extensive and painful operations, which may also cause possible infections of the donor site [24], [25]. A second option is an allograft transplantation which overcomes the problem of tissue availability because tissues are obtained from cadavers. However, the disadvantages of this approach are reduced osteoinductivity, triggering of the immune system and transmission of diseases [24], [26]. An alternative choice could be the utilization of metal implants but they have also some limitations such as degradation issues, several operations are required to remove them from transplanted site and they may potentially leak toxic ions which trigger host immune system reactions and infection [27], [28]. A third choice is synthetic materials or allografts as a candidate for bone regeneration, but they are not completely suitable for this process because host immune system sees them as foreign bodies and makes a thin fibrous membrane around them. Thus, host tissues do not accept allograft transplants properly and the process may be finalized by rejection [29]. To overcome these limitations, researchers have been working on new strategies as being approaches to tissue engineering [30].

Today, tissue engineering principals provide an ideal choice with respect to the reconstruction of bone defects. The aim of this strategy is to overcome the limitations, which are exhibited by transplantation of tissue grafts and biomaterials [30].

1.1.4 Tissue Engineering: An Orchestra of Scaffolds, Cells and Signaling Molecules

Tissue or organ failure is one of the most challenging issues in medicine. There are some traditional approaches that deal with this problem. These approaches are (1) organ transplantation from one individual to another, (2) tissue grafting from a healthy region to an affected region in the same individual, (3) providing tissue function with mechanical devices (prosthetic valves, joints, dialysis machines, etc.), and (4) pharmacologic supplementation of metabolic products of the affected tissue [31]. While the aforementioned therapies have had a significant medical impact, there are newer technologies on the horizon that seek to overcome the limitations of these traditional approaches.

One new and fast developing interdisciplinary area is called tissue engineering (TE), developed during the last three decades. TE combines biology, medicine, and engineering sciences and aims to create new tissue equivalents to damaged or lost tissues and organs by using cells, biomaterials and biologically active molecules [32], [33]. The first definition of TE was made by Richard Shalak and C. Fred Fox at Tissue Engineering: Proceedings of a workshop held at Lake Tahoe in 1988 as: “The application of the principles and methods of engineering and the life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissue and the development of biological substitutes to restore, maintain or improve functions” [34]. Then, in 1993, Langer and Vacanti summarized and defined TE as “an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain or improve tissue or organ function” [35]. Generally, TE strategy is based first on isolating cells by biopsy from the patient, growing them on a biomimetic scaffold under known and controlled culture conditions and then on implanting the final construct into the desired site in the patient’s body. Biomimetic scaffolds should provide suitable environments for host’s cells to synthesize new extracellular matrix (ECM). This newly generated ECM helps to improve tissue function [32], [33]. The presented TE

techniques are mostly dependent on the use of macro-structured porous scaffolds. These materials work both *in vivo* (living organism experiment) and *in vitro* (test plate experiment) as supports for the initial cell attachment and following tissue formation [36-38] (Figure 1.6).

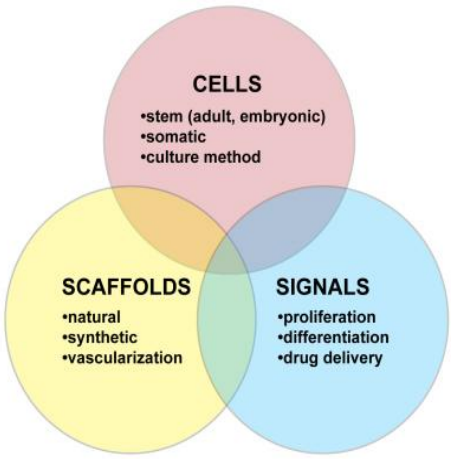


Figure 1. 5 The main triad of tissue engineering [39]

There are several crucial stages that should be taken into consideration while producing engineered tissues. According to Langer, there are three stages [40]:

1. Selection of most suitable biomaterial in the form of 2D or 3D scaffolds.
2. Selection of the most suitable method to design a scaffold to mimic the *in vivo* environment of the natural extracellular matrix (ECM).
3. Selection of the most suitable cell type for culturing in a scaffold.

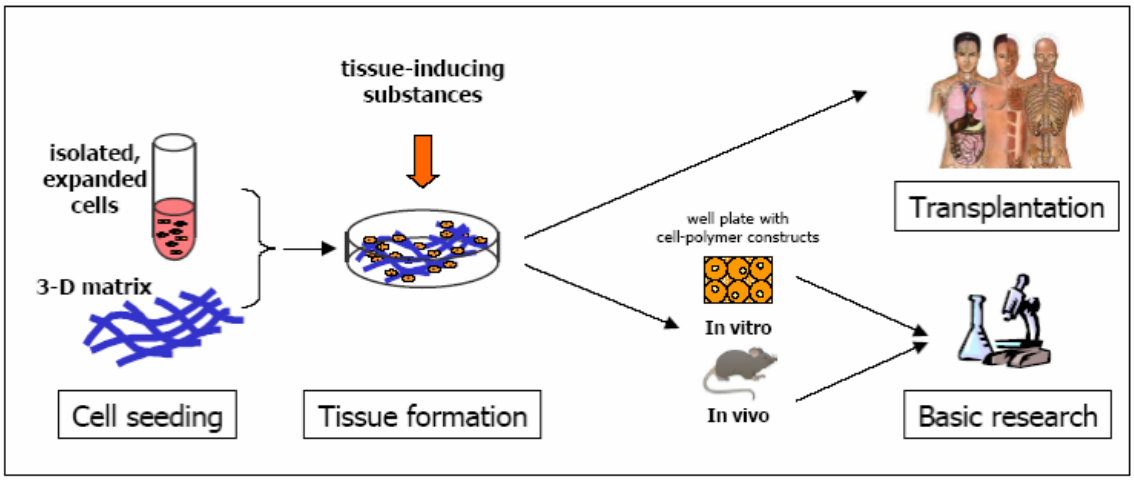


Figure 1. 6 Diagram of TE approaches [41]

The scaffold fabrication techniques affect the properties of a material and degradation characteristics of the material so choosing the suitable scaffold preparation technique is very crucial for TE applications. With a proper fabrication technique, well-designed scaffolds can be produced, and they direct cells in the desired manner. As a result, tissues and organs can be obtained with desired properties [42].

From the literature, a scaffold should contain following properties:

1. Biocompatibility: it should not cause any host tissue response [43].

2. Surface chemistry: it should allow cell attachment, migration, proliferation, and differentiation of the cells [44-46].

3. Interconnected pores: it should support cell infiltration and vascularization [47], [48].

4. Biodegradability: in controlled manner it allows for new tissue formation [49], [50].

5. Sufficient mechanical properties: it is important to keep the structure and function after implantation and during remodeling of the implants [51-53]. Moreover, it supplies a better environment for cells, transports bioactive molecules and cells to the targeted site and allows for the control structure and function of the newly formed tissues [47], [54].

6. ECM formation: it should support cellular functions and allow biomolecular signals to reach the cells [55].

7. Biomimicking: TE scaffolds should mimic the natural ECM's structure and biological function [56], [57].

1.1.4.1 Bone Tissue Engineering

Bone tissue engineering is a promising approach to eliminating problems which are caused by traditional treatments. Generally, bone tissue engineering combines an artificial extracellular matrix or scaffold, osteoblasts or cells which can differentiate into osteoblasts-like mesenchymal cells and bioactive molecules like growth factors or cytokines which regulate cell adhesion, proliferation and bone formation [58]. An

example can be seen in Figure 1.7, which describes bone tissue engineering hypothetically.

Nowadays, much of the research is focused on developing suitable 3D biomaterials as scaffolds for bone tissue engineering applications. Depending on the ideas, a convenient scaffold should enhance cell adhesion, proliferation in favored 3D orientations. Moreover, it should support cells mechanically and possess a suitable architecture regarding remodeling of bone tissues [13]. Another important factor relates to creating biodegradable materials. In addition to this, these scaffolds should allow the proper diffusion of oxygen and nutrients, and as well as waste products. All in all, the last aim is to return complete biological and mechanical properties of injured bone tissue [13].

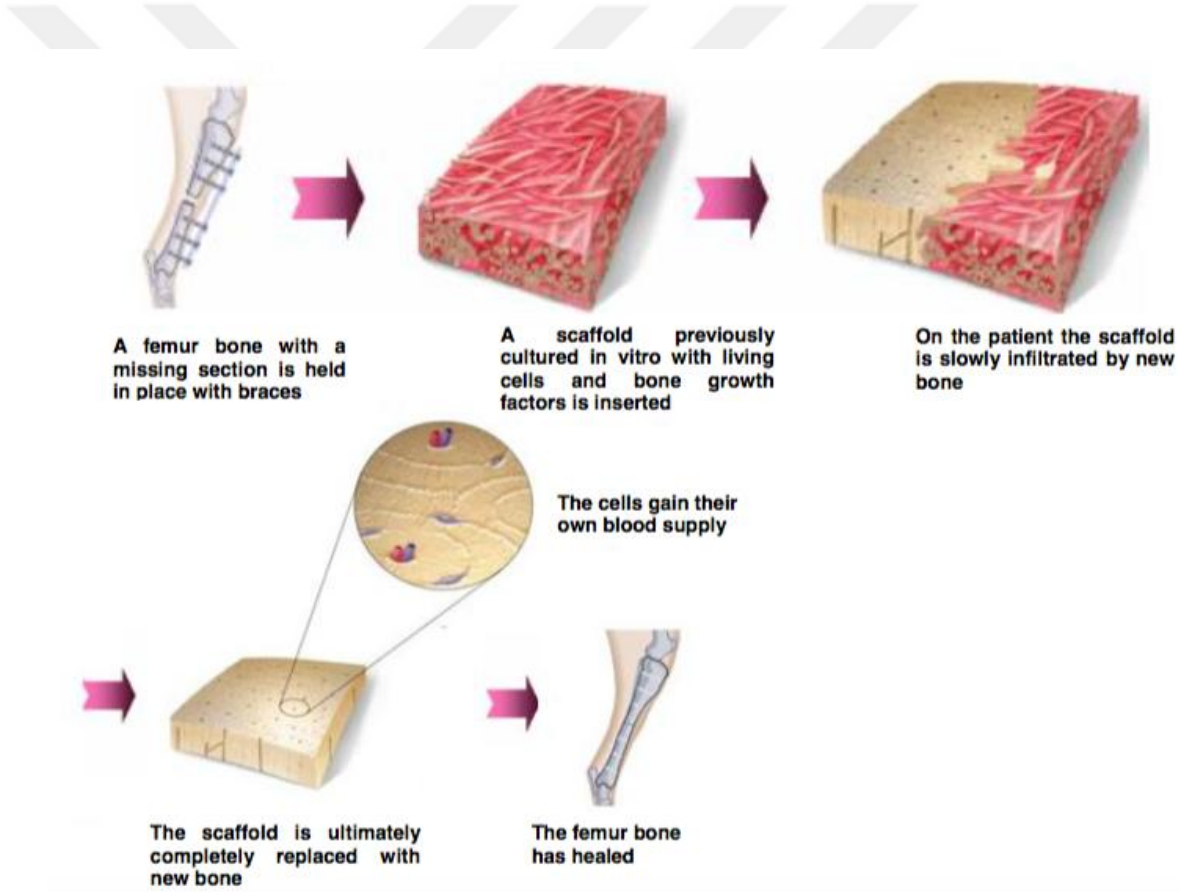


Figure 1. 7 A basic scheme of bone tissue engineering [13]

1.1.4.2 Fundamental Properties of Scaffolds for Bone Tissue Engineering

The fundamentals of a suitable scaffold for bone tissue engineering applications are complex and in many cases, there is no full agreement within the scientific community

about the requirements [59-61]. However, scientists do agree with some characteristics for bone tissue engineering scaffolds.

i. Biocompatibility

An ideal scaffold should possess a biocompatible character and its degradation products must not initiate any immune system reactions or toxicity [62-65].

ii. Desirable Mechanical Properties

Suitable mechanical properties are crucial for sustaining ideal environmental stress for the new tissues [62-66]. Moreover, the scaffold should be mechanically strong enough and stable to overcome the stress before extracellular matrix synthesis by the cells [67].

iii. Controlled Degradation Rate

Another issue of concern is biodegradability. The scaffolds should be biodegradable and the degradation rate should be controllable in order to follow cell/tissue ingrowth both *in vitro* and *in vivo* [67]. The degradation rate and regeneration speed should be compatible with each other. In other words, when a scaffold is completely degraded, the injured site should be totally regenerated [67].

iv. Proper Pore Size and Morphology

Another important factor is the transportation of nutrients in biomaterial which is related to pore size and pore structure. One the one hand, small pore diameter results in the larger surface area. On the other hand, if pore diameters are smaller than the diameters of cells (generally 10 μm), [62], [64], [65] then the cells cannot migrate and proliferate throughout the scaffold. Moreover, bone ingrowth is enhanced by larger pore diameters such as more than 100 μm [68]. Therefore, there is controversy or disagreement regarding the selection of optimum pore size for bone tissue engineering applications. Some of the researchers say the best pore size of scaffolds for bone tissue applications, ranging from 100 to 150 μm [64] whereas the remaining says 100 to 350 μm [69].

In addition to this, interconnectivity between pores is very crucial since an interconnected pore network structure allows for the diffusion of the nutrients/oxygen and also supports vascularization. Moreover, waste products can be removed out of the scaffold easily.

v. Appropriate Surface Chemistry

Another important topic is maintaining suitable surface chemistry because it enhances cell adhesion, proliferation and differentiation and additional cellular events such as spreading, interconnection, migration and biosynthetic activity [62], [64], [65]. So the materials' surface issues should be taken into consideration such as topography, chemistry, surface energy or wettability while producing biomaterials [62], [64], [65].

1.1.5 Scaffold Fabrication Methods

Selection of the correct fabrication method is one of the most important milestones in TE pathway. In the literature, there are many methods which are used to produce porous scaffolds. Some of them are mentioned in the following paragraphs.

The first technique is fiber bonding which consists of interconnected fiber networks and was prepared by Mikos et al., in 1993 [70]. In this technique, polymer fibers are put in an aligned position into a mold and then embedded in a solvent. This solvent dissolves one of the polymers, but not the other one. Then the solvent is evaporated and the polymer is heated above its melting temperature. Finally, the polymer fibers are joined at cross-points, physically.

The second technique is called phase separation. In this technique, at low temperatures, the polymer is dissolved in an organic compound such as molten phenol, naphthalene or dioxane. Then this step is followed by dispersion of bioactive molecules in these solutions [71]. By lowering the solution temperature of the liquid-liquid or solid-liquid phase, separation is generated. Then, the solidified solvent-rich phase can be removed by sublimation, evaporation or extraction [72] which produces a porous polymer scaffold.

A third method is freeze drying and this technique depends on sublimation. Firstly, the polymer is dissolved in a solvent at the desired concentration. Then the solution is frozen and the solvent is removed by lyophilization under high vacuum conditions. The resulting, scaffolds are obtained with high porosity and interconnectivity [44], [45]. Using the freeze drying method, desired pore sizes, and 3D pore homogenous structures can be obtained by changing the freezing rate and pH. A fast freezing rate results in smaller pores while a slow freezing rate produces bigger pores. On the other hand,

solidification in a single direction has been used to create homogeneous 3D pore structures [72].

The next technique is solvent casting which is based on the evaporation of a solvent to form a scaffold by one of two ways. The first method for solvent casting involves dipping a mold into a polymer solution for adequate time to form a polymeric membrane layer. In the second method, a polymer solution is added into a mold for adequate time, and the solution is then let to evaporate. As a result, a layer of the polymeric membrane is obtained [71].

The fifth one is particulate leaching which is a technique used to produce porous scaffolds. In this technique, salt, sugars or wax are used as a porogen. For example, salt is ground into desired small particles and the particles are put into a mold. Next, a polymer solution is poured into the salt-filled mold. Then the solvent is evaporated and salt crystals are leached off using water. Finally, porous scaffolds are obtained. The pore size can be controlled by the adding a different size, shape and amount of porogen [73].

The further technique is gas foaming. When using a gas foaming procedure there is no need to use an organic solvent and high temperature. This fabrication method is based on the usage of high-pressure carbon dioxide gas. The polymer is exposed to the high pressure of carbon dioxide gas to saturate it [74]. A phase separation process occurs under this condition because dissolved carbon dioxide becomes unstable and separates from the polymer. To minimize the free energy, gas molecules make clusters and pore nucleation occurs. At the end of the foaming process, 3D porous scaffolds are obtained. The scaffold porosity can be controlled by the amount of wax, salt and sugars used [75].

Finally, electrospinning is a technique which is used to fabricate scaffolds from nanoscale to microscale levels. Its principle depends on the usage of electrostatic forces to produce polymeric fibers which are supplied by two electrically oppositely charged electrodes. One of the two electrodes is set into the polymer solution and the other is set into the collector. Next, polymer solution drops are produced by a pumping force. Then, an electric field is applied to the droplets to eliminate the surface tension of the polymer solution. The polymer fiber is produced by the ejection of a jet polymer. At the same time, the solvent evaporates and the nanofibers are collected into the collector. In the literature, there are many studies which have used many polymers like silk fibroin [76-78], collagen [79], chitosan [80], gelatin [81], etc.

However, most of these techniques are dependent on complex procedures, require specific equipment, take place at high temperatures and use toxic solvents [82]. Besides these techniques, there is an additional route to produce scaffolds for tissue engineering applications which are called cryogelation.

Cryogelation is a very simple method which uses ice crystals as templates to produce macroporous structures without the addition of any organic solvents. A more detailed explanation of this method is given below.

1.1.6 Cryogelation

Cryogelation is a new route to obtaining 3D macroporous scaffolds. In this method, the polymerization reaction occurs below the freezing point of the system. The gelation process is carried out at low temperatures and hence it is called cryogelation. Cryogelation as a term that was used for the first time by Lozinsky et al. They made interconnected polymer networks [83], [84]. They also used cryogel (from the Greek κριοσ -kryos- meaning frost or ice) as a term to describe macroporous hydrogels which are produced by the cryogelation method.

Cryogelation systems are partially frozen before polymerization and ice crystals act as a template. On the other hand, monomer/polymer, crosslinker, and initiator stay in the unfrozen liquid part of the system where polymerization occurs. Next, ice crystals can be easily removed by thawing, and pore structures occur in those places. The morphology of the networks is polyhedral which builds pore walls [85], [86] (Figure 1.8).

When cryogelation is compared with the other techniques, the cryogelation method has many advantages [87]:

- Straight and ordered macropores by avoiding significant pressure drop.
- Crack-free materials in a monolithic shape.
- Inexpensive, no need to use additional templates.
- No requirement for special procedures to remove solvents like calcination and chemical etching.

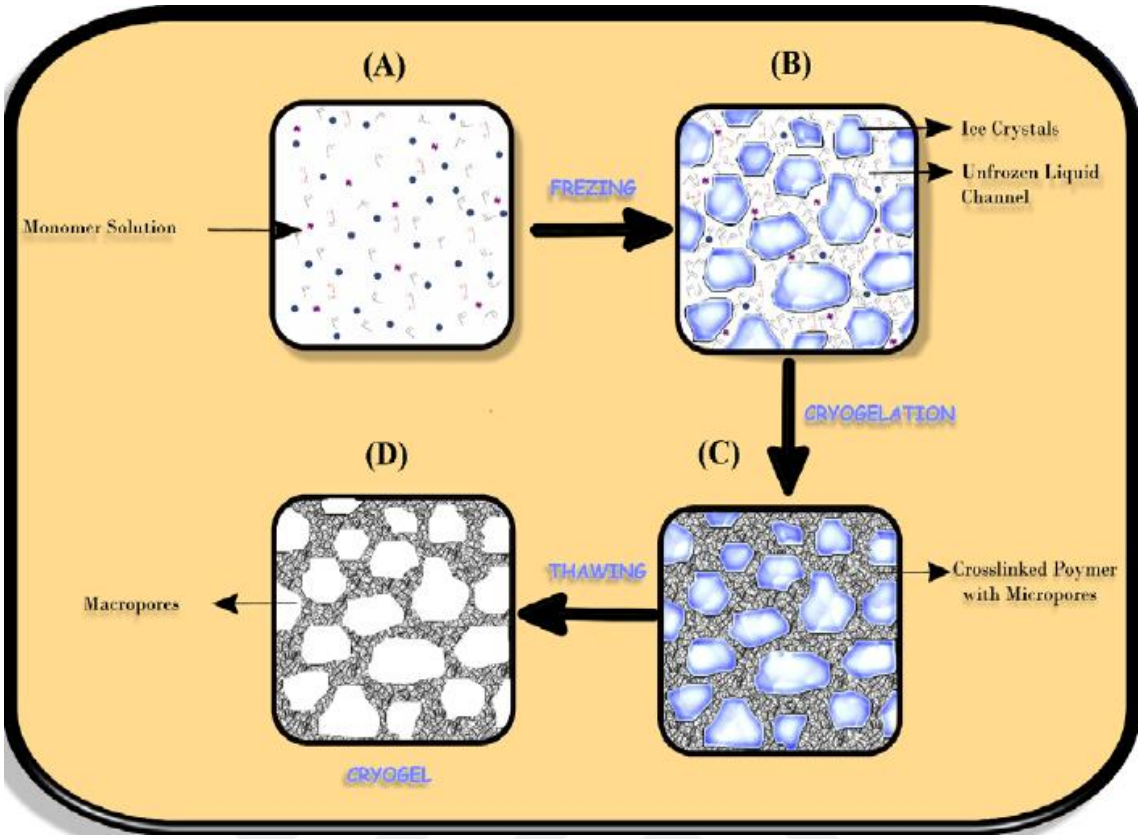


Figure 1. 8 Schematic representation of cryogel formation. A) Initial monomer solution B) Formation of crystals of frozen solvent and unfrozen liquid channels upon partial freezing C) Polymer matrix formation by cryogelation of apparently frozen system D) Formation of cryogel with macropores after thawing of the ice crystals [88]

The temperature of the reaction solution which contains monomer/polymer solution, crosslinker, the initiator should be a few degrees centigrade below the freezing point of the solvent (Figure 1.8.A). The partially frozen system is heterogeneous and contains both solid blocks and unfrozen liquid microchannels between solid crystals [89] (Figure 1.8.B). In this system, ice crystals push gel-forming agents into micro-channels. The micro-channels' volumes are lower than the total initial volume of the system. As a result, the concentration of monomer/polymer is higher in these micro-channels which promote the gel formation at low temperatures. Also, cryogelation systems require much lower monomer/polymer concentration for the gelation processes compared with other scaffold fabrication techniques [90]. On the other hand, a high concentration of monomer/polymer solution in micro-channels enhances chemical reactions. Therefore, cryogelation is much faster than the other reactions in homogeneous solutions above the freezing point of the solvent. Moreover, these microchannels contain a high concentration of precursors which causes the formation of microporous gel structures in pore walls [84] (Figure 1.8.C). The interconnected pore structure is maintained by the

solvent crystals which grow during the freezing process. The concentration of the precursors and conditions of the cryogelation process affect pore size and distribution.

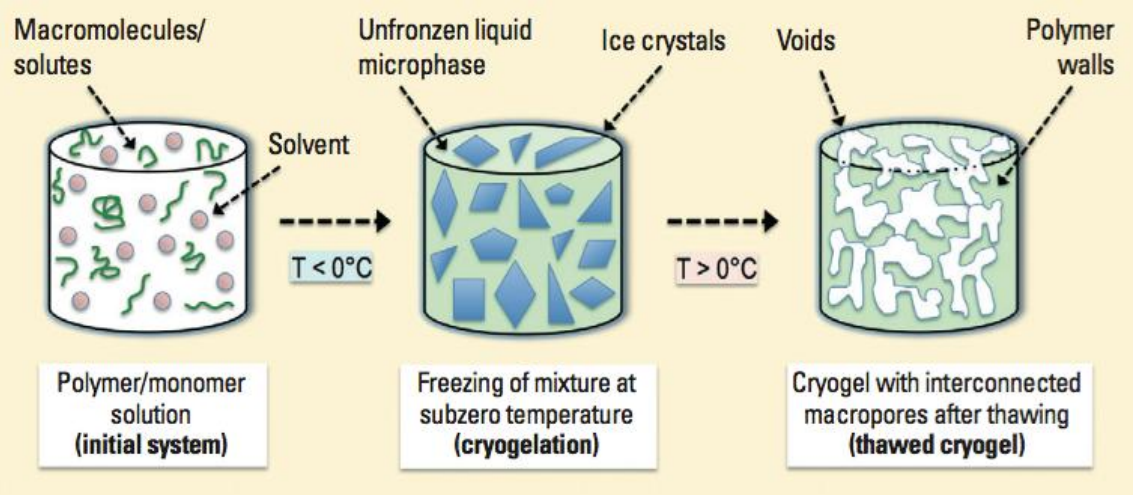


Figure 1. 9 Another illustration of the cryogelation process [91]

Cryogels have a heterogeneous phase structure because they have both a frozen solid (ice) part and an unfrozen liquid (precursor solution) part. On the other hand, cryogels also have heterogeneous porous structure. In addition to having macropores, as a result of ice crystals, they have micropore structures between the polymer chains (Figure 1.8.D). In cryogelation, the initial system should have a solvent which can be frozen. This property separates cryogelation from chilling induced gelation because cryogelation carries on without any phase separation at low temperatures [85].

Researchers who have worked on cryogels have mainly investigated the formation and properties of cryogels fabricated from different kinds of materials [92-95]. Also, most of the studies rely on the controlled porosity [92-96] and surface chemistry [97]. These studies proved that changing the initial monomer/polymer concentration of the precursor system affects the pore size and wall thickness [92]. When the monomer/polymer concentration is increased, the pore wall thickness increases. In contrast, it decreases the total porosity of the cryogel. Furthermore, the freezing rate also is an important parameter for cryogelation process. Pore size and distribution are affected by the freezing rate of the reaction system [98].

1.1.7 Cryogel Scaffolds

Recent studies have shown that cryogelation is a promising technology for producing macroporous gels which are called cryogels, for tissue engineering applications [99]. In

the literature, cryogels are used in a wide range of application areas. They are used for different purposes due to their osmotic, mechanical and chemical stability. For example, they are used in chromatography of large biomolecules such as proteins, viruses, cell organelles and whole cells. They can be used both in bead form and as a column matrix. Also, cryogels are used in cell culture and cell-biomaterial interaction applications [95], [100], [101]. Moreover, cryogels can be used for cleaning the environment. For example, some cryogels were produced as an oil absorbent which removes crude oil from water [102], [103].

As mentioned earlier, cryogels are produced at sub-zero temperatures and ice crystals cause an interconnected macroporous structure. From a TE perspective, this interconnected porous structure of cryogels promotes the exchange of beneficial molecules and waste products. Moreover, cryogel scaffolds provide a suitable environment for their growth [104]. Cryogels are fabricated from both synthetic and natural polymers. They are used to support cell adhesion, proliferation, and migration. Different kinds of cells such as chondrocytes [105], osteocytes, cardiomyocytes [106], fibroblasts [107], etc. have been cultured in these 3D cryogel structures. Many studies about cryogels in the form of monolithic rods, discs, and sheets are present in the literature [93], [94]. Cryogels can be fabricated from natural-based polymers (such as gelatin, agarose, chitosan, silk, deoxyribonucleic acid (DNA) [84], [85] etc. and synthetic-based polymers (such as polyvinyl alcohol (PVA), polylactic acid (PLA), poly(2-hydroxyethyl methacrylate) (pHEMA), polyethylene glycol (PEG), polyglycolic acid (PGA), [93], [94] and poly(lactic-co-glycolic acid) (PLGA) etc. Therefore, cryogel scaffolds can be produced from different kinds of polymers with varying mechanical and biological properties.

A recent study of interest is in one of the most popular materials, natural polymer-gelatin; which has good mechanical properties for bone TE applications [108]. Also, ceramics, especially hydroxyapatite, are under consideration for the production of bone TE cryogels because natural bone tissues contain 50% of hydroxyapatite and, therefore, hydroxyapatite supplies a good environment for bone cells. With the proper combinations of gelatin and hydroxyapatite, mechanical and biocompatibility, properties of cryogels for bone TE can be improved.

1.1.7.1 Synthetic Polymer Based Cryogels

Synthetic polymer based scaffolds have been used as ECM analogs which have very important advantages. Properties of synthetic based scaffolds can be controlled in a desired manner and also in their function. Therefore, they can be adapted to various types of cells. That is why they are used in a wide range of applications for TE. Moreover, synthetic polymer based scaffolds have good mechanical strength and known degradation rates [109].

Unfortunately, synthetic polymer based scaffolds have a drawback because of their lack of proper surface chemical properties. This problem is solved by controlling surface properties synthetically, but they do not have all the components of natural ECM [110], [111]. Moreover, some of the synthetic polymers have another disadvantage, they can produce degradation products. This process makes acidic products and when they are collected in a local part of the living tissues, this can that cause toxic effects [112].

It is seen in the literature that scientists focus on several kinds of synthetic polymers to make suitable scaffolds for tissue engineering applications. Researchers mostly use PGA, PLA, PGLA, PCL, PEG, and pHEMA synthetic polymers separately or their blends and copolymers.

There are several studies which are involved in bone TE. For example, Piskin et al. synthesized a novel dextran modified with oligo l-lactide bearing hydroxyethyl methacrylate (HEMA) cryogels. Different implantation sites including dorsal, subcutaneous, iliac submuscular, auricular and calvarial defects were tested. Good bone regeneration occurred in the cranial model. They concluded from their work that this novel scaffold is highly biocompatible and has good mechanical properties [113].

1.1.7.2 Natural Polymer Based Cryogels

Natural polymers are produced mostly from plants, animals, and microorganisms. Natural polymer based scaffolds are very suitable for tissue engineering applications because they have superior biocompatibility and biodegradability properties [114]. These properties are a result of natural polymers being biologically similar to natural ECM. The interaction between cells and scaffold occurs in a natural way which causes cell attachment, proliferation, and differentiation. However, these natural polymers have some disadvantages such as different degradation rates and poor mechanical properties

[76], [115]. Another important disadvantage of natural polymers is that they can cause an immunogenic response in the host.

The most commonly used polymer sources are derived from proteins (such as collagen, gelatin, silk, etc.), polysaccharides (such as starch, dextran, agarose, chitosan, hyaluronic acid, etc.) and polynucleotides (such as DNA and RNA). Due to their nature, biopolymers (natural-based polymers) are of interest in TE applications. In addition, cryogels are smart and excellent materials as a scaffold so production of cryogels from natural-based polymers is being investigated. .

There are many studies of natural-based cryogels in the literature. For example, Fassina et al. produced gelatin-based cryogels and seeded them with human SAOS-2 osteoblasts. They then applied ultrasound to the culture system to cope with some culture problems such as limited diffusion of cells and inhomogeneous cell-matrix distribution. Their result showed that applying a physical stimulus enhances bone regeneration [116]. Kumar et al. synthesized novel chitosan, agarose and gelatin cryogels. They seeded them with different cell types of fibroblasts and cardiac cells. The results showed that these scaffolds are suitable for cardiac and skin tissue engineering applications [117]. Another study was undertaken by Rodrigues et al. who produced collagen-nanohydroxyapatite scaffolds to repair bone defects. They synthesized collagen-nanohydroxyapatite cryogels in different amounts such as 70:30; 50:50; and 30:70. Cell culture studies showed that cells attached and proliferated in all cryogels surfaces. They showed that cell proliferation was higher in nanohydroxyapatite added cryogels compared to controls without nanohydroxyapatite cryogels. They concluded their study as collagen-nanohydroxyapatite cryogels have potential to enhance bone regeneration [118].

i. Gelatin Cryogels

In the Food Chemicals Codex, gelatin is defined as “the product obtained from the acid, alkaline, or enzymatic hydrolysis of collagen, the main protein component of the skin, bones, and connective tissue of animals, including fish and poultry.” Gelatin does not exist in nature freely and cannot be obtained from non-collagen containing parts of the vertebrates such as horns and hoofs. Also, it has no plant source, even if it is described as vegetable gelatin such as seaweed extracts [119]. Collagen is composed of all 20 amino acids. All of the collagens share certain and common characteristics despite there being some differences in amino acid composition. It contains the highest amount of

hydroxyproline and hydroxylysine when compared with all other mammalian proteins. Gelatin contains 19 amino acids and they are linked by peptide bonds. It is composed of mainly glycine (30%) and proline/hydroxyproline (25%), alanine and glutamic acid [119]. The chemical formulation of gelatin is similar to its parental collagen which is degraded from and characterized by repeating Gly-X-Y sequences. X is mostly proline and Y is mostly hydroxyproline [108]. The chemical structure of gelatin and its amino acids are presented in Figure 1.10.A and Figure 1.10.B.

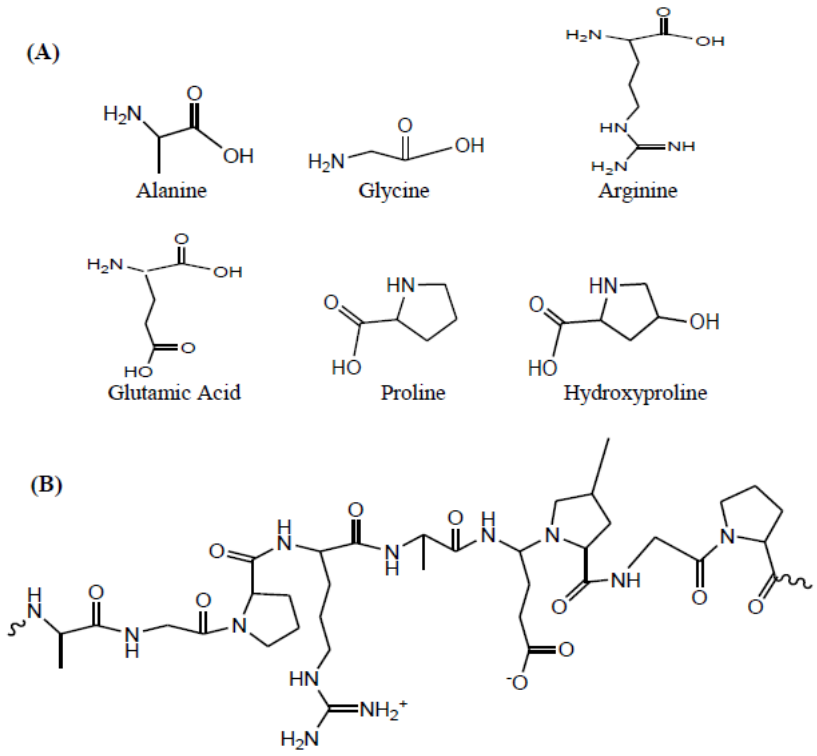


Figure 1. 10 (A) The chemical structure of amino acids of gelatin (B) The chemical structure of gelatin [108]

Collagen has a triple helix structure which forms fibers of connective tissue matrix. If an acid or alkaline effect is applied to collagen, it loses its fibrous structure and its covalent bonds are broken irreversibly. Moreover, collagen can be denatured in hot water due to the breakdown of hydrogen bonds and electrostatic bonds between the triple helix structure. In this way, several gelatin molecule chains can be produced [120]. Next, a cooling process is applied to produce cross-links or junction zones with the help of partially formed triple helix structures [120].

Gelatin is brittle like a glass material and yellowish in color and is nearly tasteless and odorless. Its density is around 1,3-1,4. It is soluble in water, however, at high concentrations, it requires heat to be solubilized. The gelatin solution's behavior is

influenced by temperature, pH, method of manufacture, thermal history, and concentration [119]. There are three kinds of gelatin sources; fish [121], bovine [122] or pork [123]. There are two ways to obtain gelatin from collagen and therefore, it is called type A or type B gelatin. If gelatin is produced by an acidic pretreatment, it is named as Type A gelatin. If it is processed by an alkaline pretreatment, it is named Type B gelatin. In alkaline treatment, glutamine and asparagine residues are converted into aspartic acid and as a result, type B gelatin contains a higher amount of carboxylic acid relative to type A gelatin and which makes type B gelatin more negatively charged [124]. Therefore, it is better suited to interactions with positively charged molecules [125].

Gelatin is one of the most famous biopolymers which is used in a wide range of areas like food, medical, pharmaceutical, cosmetics and photographic applications because of its good and functional properties [126]. In addition to this, gelatin is a cost efficient and readily available material. Also, gelatin is a polymer which is a safer biopolymer than collagen. Collagen has some limitations such as potential pathogen transmission, immune rejection, limited handling, insufficient mechanical properties, and poorly controlled biodegradability [127].

Gelatin has been used for many years as a vascular prosthesis, wound dressing, and drug delivery carrier material. And finally, it has generated interest in tissue engineering applications [115], because it has excellent biocompatibility and biodegradability properties. Moreover, it has the Arg–Gly–Asp (RGD)-like sequence which increases cell attachment on the gelatin-based scaffolds [55]. Being a water soluble material, gelatin is crosslinked by different agents like glutaraldehyde [99], genipin [128] or carbodiimides [129]. It has been shown that gelatin scaffolds are promising materials for tissue engineering. In many studies, using different types of cells like nerve [114], cartilage [130], cardiac [131] and skin [132], and gelatin showed good adhesion, migration, and proliferation on gelatin scaffolds. Guilak et al. showed good results for adipose derived and muscle-derived stem cells on gelatin-based scaffolds and suggested these materials for cartilage and bone tissue engineering applications [133], [134]. In another study, Kim et al. conducted gelatin scaffolds with nano-hydroxyapatite particles. Moreover, they reported that gelatin scaffolds with nano-hydroxyapatite modification stimulate osteoblast adhesion and proliferation [135]. Chen et al. prepared elastic macroporous gelatin/chondoitin-6-sulfate/hyaluronan (GCH) cryogel scaffolds

for cartilage tissue engineering. Furthermore, they incorporated chitosan into cryogel replacing 20% of gelatin to produce CGH-chitosan cryogels and compared CGH and CGH-chitosan cryogels for mechanical properties and chondrocytes response. They reported that macroporous elastic cryogels can be produced from ECM components with similar compositions. Moreover, they demonstrated that CGH-chitosan cryogels provided better physical/mechanical properties and also showed functional repair of defects in rabbits. Thus, they concluded from their study that CGH-chitosan cryogels are suitable biomaterials for cartilage tissue engineering applications [136]. In another study, Kumar et al. fabricated 3D scaffolds by blending a synthetic polymer-poly (ethylene glycol) (PEG) and as a natural polymer gelatin via cryogelation method and using two different crosslinkers: glutaraldehyde and EDC-NHS. They showed that PEG-gelatin cryogels presented interconnected porous structures, good physical/mechanical strength and suitable biodegradation properties. Moreover, they demonstrated that PEG-gelatin cryogels provided good micro-environments for different cell types like IMR-32, C2C12, and Cos-7. Thus, they said that PEG-gelatin cryogels were suitable for different tissue engineering applications like neural, cardiac and skin [137].

Furthermore, there are commercial gelatin-based tissue constructs available and produced by Pfizer, used as homeostatic absorbable devices for neuro, thoracic and ocular surgery [138].

ii. Ovalbumin Cryogels

When considering a material for bone tissue engineering, we can see that the egg white protein ovalbumin (OVA)-has not been studied very well in comparison to many other natural and synthetic polymers [139], [140]. Nowadays, scientists have been attracted to OVA based biocomposites for bone TE applications [141], [142] because the OVA based biocomposites are cost-effective, highly available, have good interaction with native tissues and their by-products after degradation in body fluids, are safe [143].

The chicken egg shows great potential for bone tissue engineering approaches. Interestingly, the chicken egg shell's proteins are similar to collagen- the most abundant protein in bone tissue. In the eggshell, albumen works like collagen, takes place in calcium carbonate formation and precipitation of calcium phosphate for mineralization [144]. This information highlights that albumen and collagen have similar effects in their hosts [140]. Also, 54% of albumen by weight, consists of globular

phosphoglycoprotein OVA and its molecular mass is about 45 kDa [144]. The OVA comprises 386 amino acids and shares many of them with human serum albumin [144]. Half of the amino acids in the OVA are hydrophobic and one-third is charged. Most of its parts are acidic such that it has 4.5 isoelectric points [145]. According to Stein and his friends' work, OVA contains 30% alpha helix and 32% beta sheet structures [146], [147]. This result was in an agreement with Egelandsdal's work who found, in 1986, that OVA is made up of 30% alpha helix and 40% beta sheet structure. Although these results were a little bit different to Painter and Koenins's studies, who found 49% and 25% α-helix respectively and 13% and 25% β-sheet structures, respectively, in 1976. These differences could be due to experimental conditions like ionic strength and pH [148].

OVA molecule contains four sulphydryl groups, three of them are weakly reactive in the native form but the fourth, is reactive in the denatured OVA [149-151]. Because of its homologous sequences, OVA was classified as a member of the serpin superfamily. The serpins are a family of proteins with more than 300 homologous amino acid sequences with different functions in animals, plant, insects, and viruses [152], [153]. They function as a protease inhibitor. However, even OVA is classified in serpin superfamily, and in its native form, it lacks protease inhibitory activity [146], [154-156]. Interestingly, in 1996, Mellet et al. showed that when OVA is heated at 97^{o}C, for 30 minutes, it undergoes conformational changes and becomes an inhibitory protein of some proteases. However, this inhibitory ovalbumin protein is different from other serpin family proteins, because it does not contain the information required to form irreversible complexes with proteases [157]. Because of this characteristic, many researchers have said that OVA is a storage protein [158]. Therefore, in many developing embryos, the OVA changes its structure to a less ordered form which is suitable for the migration. So that means OVA may have a dynamic function in developing organs [159].

In the literature, OVA based studies for bone TE applications are very limited. Most of them focus on improving the mechanical properties of OVA scaffolds with nanocrystals and filling materials, like cellulose nanocrystals [139], [144] and hydroxyapatite [142] using mostly freeze drying [139], and salt leaching [141] methods.

When the cryogelation method is considered, it is clearly seen that there is the need for developing OVA cryogels. The first study of OVA cryogels was by Lozinsky et al. in

1997 [160]. They chose urea-induced cryogelation of OVA as a model to understand cryotropic gelation of water-soluble, cryoresistant proteins. They also wanted to see the effect of the freezing regimes and frozen sample storage on structures and properties of cryogels [160]. They showed the gelation of OVA is directly relevant to the concentration of protein and low molecular weight additives, temperature, rate of freezing and pH. They also added to their conclusion that changing the phase state of the frozen system affects the structure and property of the cryogels. Another study was undertaken by Mattiasson et al. who showed non-covalently gel formation of OVA [161]. When OVA is frozen in a urea solution, the protein unfolds and undergoes hydrophobic and electrostatic interactions that allow gel formation. Their aim was to show the differences between enzymatically cross-linked, chemically cross-linked and non-covalently formed gels. For this purpose casein was chosen, because it is a good substrate for transglutaminase (Tgase) [162], [163]. For chemical crosslinking, gelatin and glutaraldehyde and for non-covalently interaction OVA and urea solution were chosen. They tested gels mechanically and found that OVA cryogels had the highest elastic modulus. They also tested gels with fibroblasts and myoblasts. The results indicated that all the scaffolds were suitable for cell culturing. Both fibroblast and myoblast cells were viable and proliferated on the scaffolds. They proposed casein cryogels for skeletal tissue engineering applications, gelatin cryogels for softer tissues and OVA cryogels for elastic tissues [161].

1.1.8 Biocomposite Cryogel Scaffolds

Polymers, bioceramics, and metals have been used to design suitable and smart scaffolds for desired TE applications. In order to produce chemically, physically and mechanically excellent scaffolds, two or more materials are used in different combinations. These combined materials systems are called composites. There are two main ways to obtain composite materials:

1. One or more polymers are combined with a ceramic
2. Two polymers are combined.

The idea of making composite materials is to improve the polymer's and ceramic's mechanical properties. In the literature, there are many studies that use one or two polymers combined with ceramics. The most commonly used ceramic used in TE

applications is hydroxyapatite. Hydroxyapatite is used with both natural polymers, such as chitosan [164], as well as synthetic polymers, like PCL, PLA, PLGA.

In the literature, biocomposite cryogels have been synthesized in different polymer/ceramic compositions. For example, Kumar et al. combined microparticles into a polymeric cryogel which was called a cryogel-microparticle (C-MP) composite. Three different types of cryogels were produced from different types of polymers. For example, they made combinations of chitosan-agarose, chitosan-agarose-gelatin, and chitosan-gelatin. Then they combined these cryogels with microparticles (PLGA, chitosan, and silica) and investigated factors which improved homogeneous microparticle distribution. Moreover, mouse embryonic fibroblasts (NIH 3T3), mouse myoblasts (C2C12), and human lung cancer cells (NCI-H460) were seeded on both C-MPs and nonstructured cryogels. They compared cell adherence, growth, and biocompatibility and also loaded silica MP-chitosan–gelatin microstructured cryogel composites with doxorubicin to test the drug delivery capacity of the material. They concluded from their study that this material had an anti-proliferative effect on human cervical cancer cells (HeLa) [165].

Furthermore, McGuinness et al. produced PVA cryogels with chitosan, gelatin, and starch. They looked at different cryogels' mechanical properties and also analyzed cell attachment and proliferation characteristics of each cryogel blends. They concluded that the most cell attachment and proliferation was observed in PVA/gelatin cryogels. It was proposed that these produced cryogels can be used for the production of artificial blood vessels [166].

Another study was carried out by Kumar et al. They synthesized porous inorganic/organic biocomposite scaffolds using the cryogelation technique. They produced PVA–tetraethylorthosilicate–alginate–calcium oxide (PTAC) composites. They evaluated these cryogels for bone tissue engineering applications. Moreover, they compared cryogels with their previous work's products which were foams and pellets. Additionally, a hydroxyapatite-like layer was formed on the surfaces of biocomposite materials when they were incubated in a simulated body fluid (SBF). Biocompatibility tests were done using L929 fibroblasts and to observe the proliferation of MG-63 osteoblast-like osteosarcoma cells and they followed two different procedures to see the influence of SBF. Biocomposite scaffolds were both pre-soaked in SBF and non-soaked. They compared the results and they showed that pre-soaking affected cell

proliferation in a negative way. Furthermore, human osteoblasts were seeded on these scaffolds. In the early stage of the culturing, it was observed that there were cell sheets with a thick collagenous extracellular matrix on the surfaces of biocomposite cryogels. They also recognized that calcium phosphate-like mineral deposits on the surfaces of biocomposite cryogels. It is suggested that these results indicate that the PTAC biocomposite cryogels produced are good candidates for bone tissue engineering applications [167].

1.1.9 Hydroxyapatite

Hydroxyapatite is one of the most similar compounds to mineral phase of the bone tissues with the formula of $Ca_{10}(PO_4)_6(OH)_2$. In hydroxyapatite crystals, $(PO_4)^{3-}$ molecules are found in the tetrahedral form and hydroxyl (OH^-) groups exist parallel to C-axis [168]. Moreover, Ca^{2+} ions are located around OH^- and the center of the each triangular prism. (Figure 1.11) Carbonated-calcium and phosphates are the main components of the bone tissues. Calcium phosphates compounds are found in the bone around 30-40% within an organic matrix which is mostly collagen and found close to between 34-42% [168] (Table 1.1).

Table 1. 1 Physical properties of hydroxyapatite [168]

Properties	Typical values
Density (g cm^{-3})	3.15
Young's modulus (GPa)	85–90
Knoop hardness (MPa)	3450
Tensile strength (MPa m^{-2})	120
Poisson coefficient	0.3
thermal expansion ($\times 10^{-6}$ K^{-1})	11
Melting point (°C)	1660
Specific heat (cal g^{-1} K^{-1})	0.15
Thermal conductivity (W cm^{-1} K^{-1})	0.01

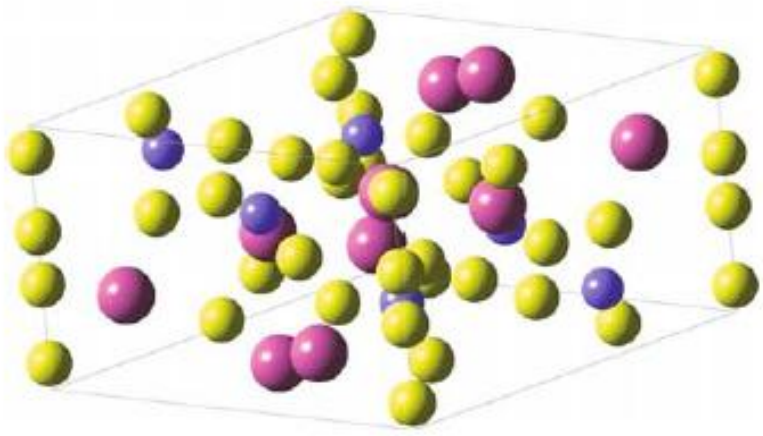


Figure 1. 11 The schematic drawing of crystal sturucture of a hyroxyapatite cell where yellow atoms are OH^-, pink are calcium, and blue are phoshphorus atoms [168]

Pure hydroxyapatite powder has a white color, but it exists in yellow, brown and green (hydroxyl proper). It can be produced by several kinds of methods including dry and wet methods (precipitation), hydroxy thermal methods and mixed preparation combined with a mechanochemical milling treatment [169]. The wet method is the most popular method to produce hydroxyapatite. In this method, under controlled pH conditions, slow precipitation occurs between calcium and phosphate precursors. In the hydrothermal method, high temperature and pressure are used to synthesize hydroxyapatite. Generally, hydroxyapatite synthesis is conducted with a sintering process which means a heat treatment is applied between 800-1000^oC to enhance the crystallinity of hydroxyapatite [170]. Oliveria et al. reported that 3D porous scaffolds, combined with sintered hydroxyapatite compounds showed good biocompatibility and bioactive properties towards mouse lung fibroblasts [171]. However, when sintering is applied, high crystalline hydroxyapatite is obtained which decreases the biodegradability. In the literature, it was shown that non-sintered hydroxyapatite based bone constructs showed better results for cell and tissue growth when compared to sintered hydroxyapatite compounds [172]. Another important property of hydroxyapatite is that it is osteoinductive [173] and as a result, it has attracted attention in bone tissue engineering studies [174].

Hydroxyapatite is found in different particle sized forms and they are widely used to improve bone regeneration and to coat surfaces of a metal prosthesis to enhance biological properties. There are many studies about hydroxyapatite showing enhanced bone regeneration in orthopedic, dental and maxillofacial application areas. It is mainly

used to improve mechanical properties of polymeric scaffolds. Piskin et al. produced gelatin cryogel scaffolds by the cryogelatin method and reported that these cryogel scaffolds had good mechanical properties when they were loaded with hydroxyapatite particles. Gelatin/Hydroxyapatite composite cryogel scaffolds demonstrated suitable behaviors for bone TE applications, especially in the craniofacial area [175]. Another study showed chitosan/nano-hydroxyapatite composites displayed better osteoblast cell differentiation and bioactivity than chitosan sponges, without hydroxyapatite particles [176]. In another recent study, synthesized chitosan hydrogels were immersed in a calcium and phosphate solution to obtain hydroxyapatite molecules by the wet method. These chitosan/hydroxyapatite membranes showed good biocompatibility for MG63 osteosarcoma cells [177]. In another novel study, scaffolds were obtained from phoshonated OVA and immersed into precursors of hydroxyapatite. Moreover, these scaffolds were combined with MC3T3-E1 mouse preosteoblastic cells. Live/dead staining and MTT cytotoxicity measurements were done to look at the biocompatibility and effect of the material on the cells. They showed that cells survived on the scaffolds and concluded from their study that Ca-P mineralization is not directly toxic to osteoblast cells [178].

1.1.10 Cells in Bone Tissue Engineering

As in other types of TE studies, choosing the correct type of cell is very crucial for a successful bone TE study. Osteogenic cells are used in two ways for bone TE applications: (1) These cells are seeded onto a suitable scaffold and then transplanted into the defect site. (2) Cells are influenced by host's osteoinductive signals. Mesenchymal stem cells, bone marrow stromal cells, periosteal cells and osteoblasts have been used to regenerate and generate bone tissues [179-182]. As explained by Kneser et. al, there are some points regarding cells that should be taken into consideration. These are isolation and expansion efficiency of cells, the stability of osteoblastic phenotype, in vivo bone formation capacity and long-term safety [181].

Until now, researchers seeded several osteocyte cell lines, somatic and embryonic stem cells on different kinds of cryogels for bone TE applications. Fassina et al. produced gelatin cryogels and the cryogels were seeded with human SAOS-2 osteoblasts. They applied ultrasound to improve culture conditions and heterogeneous cell-matrix distribution. They concluded from their study that ultrasound stimulation enhances *in*

vitro bone formation [116]. Lozinsky et al. made cryogels and they used perfusion and static cell seeding methods to add bone marrow mesenchymal stem cells. During the experiment, they observed osteogenic differentiation. They compared the two cell seeding systems and concluded from their studies that a perfusion system provides rapid and uniform distribution of cells [183]. Another study was carried out by Bolgen et al. They synthesized lactide-dextran cryogels. Then they seeded one group of cryogels with rat mesenchymal stem cells (MSCs). Another group was not seeded with the cells. Both groups were implanted into rats. Another control group used in the experiment involved the injection of rat MSCs directly into the defect. They observed new tissue and blood vessel formation and mineralization in all groups, however, the most successful results were obtained in the groups in which they used cryogels with cells [183].

For bone tissue engineering applications, primary osteoblasts or cell lines like MG63 are commonly used in vitro to screen the cell-material interaction which is easier to follow than the more complex, *in vivo* model. These cell lines act as osteoblasts and they are readily available. Moreover, they are more durable to stress conditions than primary cells. However, their proliferation profile is different than primary cells. In contrast, primary cells are not easily available and they show phenotypic differences in each passage of cells [184].

i. Human Osteosarcoma Cell Line (MG63)

Being a tumor cell line, MG63 osteoblast-like cells act like osteoblasts [185] and they have been well known and widely used for characterization of biomaterials [186]. The secondary cell lines such as MG63 provide the absence of individual variability; show improved repeatability and reproducibility [185]. Thus, the MG63 cell line is an appropriate candidate for testing tissue-engineered biomaterials *in vitro* when looking at factors such as biocompatibility, cell adhesion, spread and proliferation [187].

ii. 10T1/2 Mouse Embryonic Mesenchymal Cell Line

The 10T1/2 cell line is a clonal cell line and model for primary bone marrow mesenchymal stem cells [188], which is derived from mouse embryos in the days 14^{th} to 17^{th}. Even after long culture periods, the cells preserve their morphology. At the early levels of confluent, they show fibroblastic-like morphology and when they become confluent cultures, they produce a flat, regular and epithelioid-like layer. Furthermore, even when the number of passages reaches a high number, they maintain their

morphology and structure [189]. Moreover, the 10T12 cell line can undergo osteoblastic, myogenic, chondrogenic and adipogenic differentiation [190-193]. Changing culture medium ingredients can easily differentiate these cells. For example, if the cells are grown under the high levels of transforming growth factor beta-1 [194] or bone morphogenic protein-2, they differentiate into chondrocytes [195]. In addition, due to their homogeneity, rapid growth rate, low cost and being safe, they are commonly used as model cell lines. As a result, these advantages of 10T1/2 cells make them suitable cell line for cell culture processes [189].

As mentioned above, 10T1/2 cells are combined with many different studies and enhance our understanding in this area such as healing an osteochondral or a bone fracture defect, a tissue-engineering scaffold and some anti-cancer drugs such as vinblastine [194].

1.2 Objective of the Thesis

The purpose of this thesis is to produce new, natural polymer-based, biocompatible and biodegradable scaffolds for TE applications and characterize the produced biomaterials as potential TE scaffolds.

1.3 Hypothesis

In this study, it was predicted that the obtained macroporous ovalbumin/hydroxyapatite biocomposite scaffolds will exhibit enhanced mechanical and biocompatible properties and hence, can be considered as suitable biomaterials for tissue engineering applications.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Ovalbumin

Ovalbumin from chicken egg white was purchased from Sigma, Germany. The molecular weight of ovalbumin is 44287 Da and its solubility is 50 mg/ml in water. The ovalbumin solution was prepared by dissolving the desired amount of ovalbumin in 10 ml distilled water (Millipore).

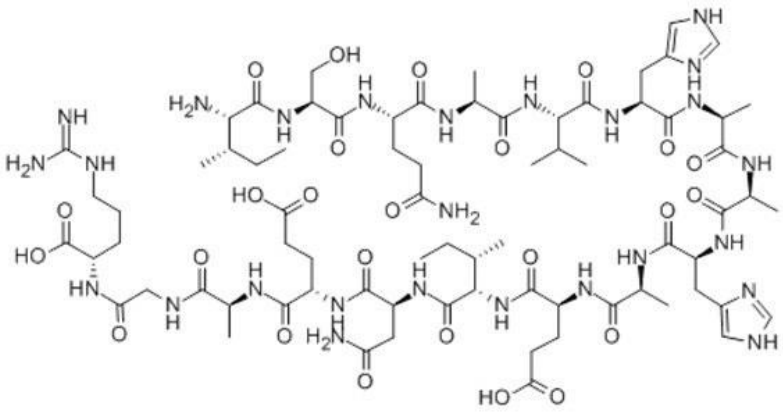


Figure 2. 1 The chemical formula of ovalbumin [196]

2.2 Solvents

2.2.1 Distilled Water (Millipore)

Distilled water was used to synthesize gels. Moreover, it was used in swelling-de-swelling experiments and to wash the gels.

2.2.2 Acetone (Merck, Germany)

Acetone was used to dehydrate the gels and also during swelling-deswelling experiments.

2.3 Reinforcing Agent

2.3.1 Hydroxyapatite (Alhenia, Switzerland)

Hydroxyapatite crystals were obtained in powder form and its color was white. Particle size measurements and scanning electron microscopy image of hydroxyapatite powder are shown in Figure 2.4 and Figure 2.5.

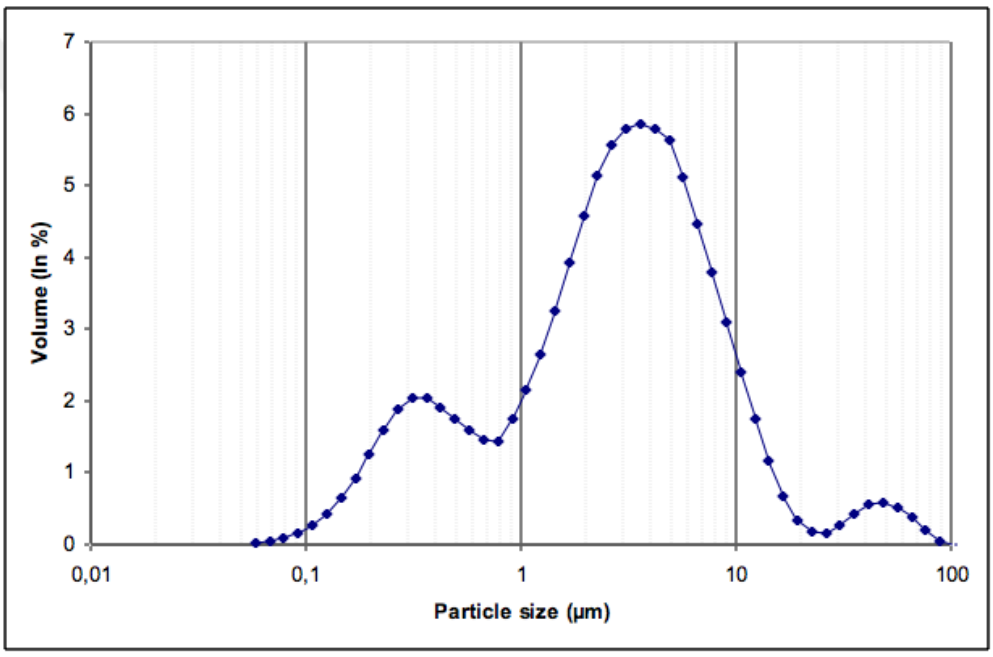


Figure 2. 2 Particle size measurements of hydroxyapatite [197]

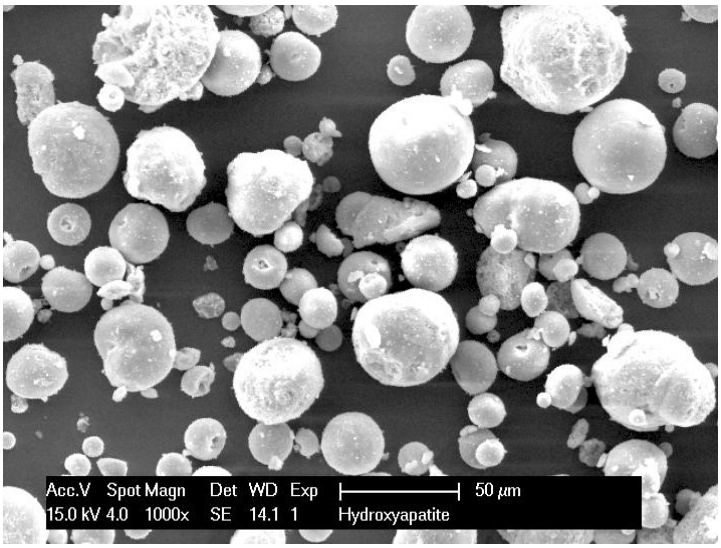


Figure 2. 3 Scanning electron microscopy image of hydroxyapatite [197]

2.4 Additives

2.4.1 Urea

Urea was incorporated into the reaction system as a physical crosslinker. It was purchased from Galenik Ecza, Turkey. The molecular weight of urea is 60,06 g/mol and was used to partially denature ovalbumin since ovalbumin is a naturally cryoresistant protein.

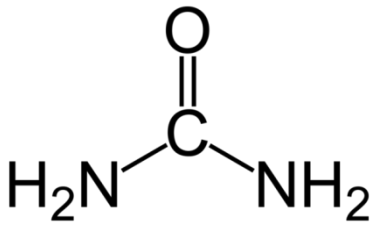


Figure 2. 4 Structural formula of urea

2.5 Equipment

- Cryostat (Kerman, Turkey)
- Freeze dryer
- Digital compass (Mutitiyo, Japan)
- 5 ml injection syringes (Set, Turkey)

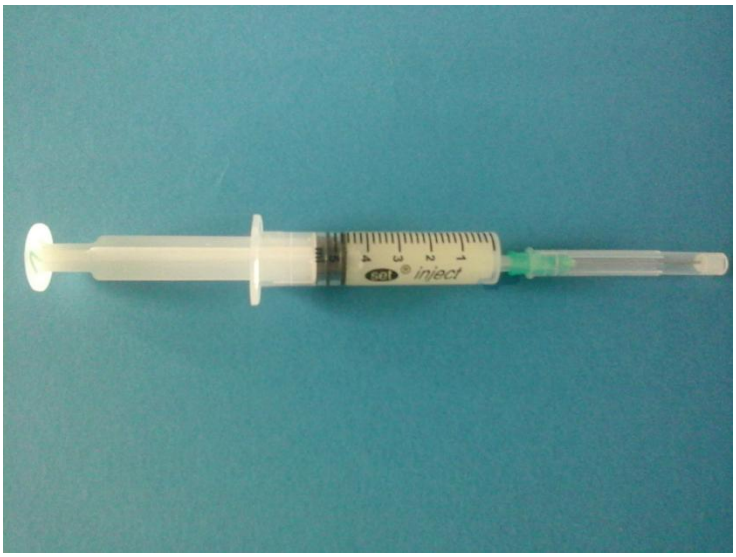


Figure 2. 5 An image of an injection syringe just after preparation filled with frozen polymer solution

2.6 Preparation of Ovalbumin Cryogels

Ovalbumin cryogels were synthesized by varying some parameters. These parameters were given in Table 2.1.

The preparation procedure of ovalbumin cryogels is given in the following steps:

Ovalbumin cryogels were prepared non-covalently using urea. 0.1 to 0.5 g of ovalbumin was dissolved in 10 ml of de-ionized water. The solution was prepared by gentle hand shaking. Then, 0.6 g of urea was added into solution and hand shaking was continued until all the urea beads were disappeared. Next, the mixture was taken into plastic 5 ml Set injection syringes (diameter 12,50 mm) and incubated in the cryostat at -12^{o}C for 24 hours. (Figure 2.7) The gels were removed from the cryostat after the reaction was completed and were thawed in de-ionized water at room temperature. Then gels were washed at least three times with distilled water. Finally, the gels were lyophilized at -45^{o}C overnight in a lyophilizer and stored at room temperature until use.

For the second set of ovalbumin cryogels, 0, 0042, 0,0065, 0,0125, 0,0250, 0,0500, 0,0750 and 0,1 g of hydroxyapatite powder was added into 10 ml of de-ionized water in different beakers. They were kept stirring at least 1 hour and after 1 hour, 0,5 g of ovalbumin was added into all hydroxyapatite suspensions separately and shaken by hand. Then 0,6 g of urea was added into the solution and hand shaking was continued until all the urea beads were disappeared. Finally, the same steps were followed as mentioned above.

Table 2. 1 The different procedures for preparation of ovalbumin cryogels and cryogel/hydroxyapatite cryogels

The changed parameter: Ovalbumin concentration (g/ml)
1% g/ml (0.1 g) ovalbumin + 6% g/ml urea (0.6 g)
2% g/ml (0.2 g) ovalbumin + 6% g/ml urea (0.6 g)
3% g/ml (0.3 g) ovalbumin + 6% g/ml urea (0.6 g)
4% g/ml (0.4 g) ovalbumin + 6% g/ml urea (0.6 g)
5% g/ml (0.5 g) ovalbumin + 6% g/ml urea (0.6 g)
The change parameter: Hydroxyapatite amount (g)
5% ovalbumin g/ml (0.5 g) + 6% g/ml urea + 0.042 w/v % HA (0.0042 g)
5% ovalbumin g/ml (0.5 g) + 6% g/ml urea + 0.065 w/v % HA (0.0065 g)
5% ovalbumin g/ml (0.5 g) + 6% g/ml urea + 0.125 w/v % HA (0.0125 g)
5% ovalbumin g/ml (0.5 g) + 6% g/ml urea + 0.250 w/v % HA (0.0250 g)
5% ovalbumin g/ml (0.5 g) + 6% g/ml urea + 0.500 w/v % HA (0.0500 g)
5% ovalbumin g/ml (0.5 g) + 6% g/ml urea + 0.750 w/v % HA (0.0750 g)
5% ovalbumin g/ml (0.5 g) + 6% g/ml urea + 1 w/v % HA (0.1 g)

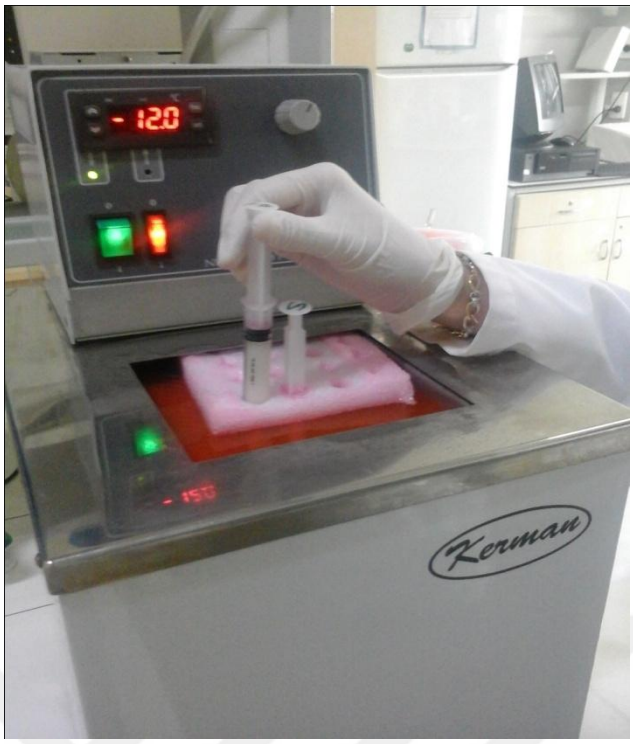


Figure 2. 6 The incubation of the gels in the cryostat at -12 ^{0}C

2.7 Characterization Methods

2.7.1 Equilibrium Swelling Measurements

The swelling measurements of the gels were carried out in distilled water. After 24 hours of the reaction in the cryostat, they were taken out and cut into sections of about 10 mm length. Next, the gels were immersed in an excess of water for at least one week to reach the swelling equilibrium at room temperature and the water was refreshed several times.

The swelling ratios of the gels were measured both volumetrically and gravimetrically. For volumetric measurements, a digital compass was used. The diameter of the gel after preparation D_0, and, the diameter of the gel after swelling were measured D. The equilibrium volume swelling ratio V_{eq} was calculated as:

$$V_{eq} = \frac{V}{V_o} = \left(\frac{D}{D_o}\right)^3 \tag{2.1}$$

where V is the volume of swollen gel and V_0 is the volume of the gel after preparation. For the gravimetrical measurements, a digital balance was used.

Moreover, the equilibrium volume and weight swelling ratios of the gels with respect to their dry state were calculated as:

$$q_v = \frac{V}{V_{dry}} = \left(\frac{D}{D_{dry}} \right)^3 \tag{2.2}$$

$$q_w = \frac{m_{sw}}{m_{dry}} \tag{2.3}$$

where V_{dry} is the volume of the dry gel and D_{dry} and m_{dry} are the diameter and the mass of the dry gels.

2.7.2 Gel Fraction Calculations

The gel fraction, W_g, defined as the amount of cross-linked (water insoluble) polymer network obtained from 1 g of polymer was calculated by the following equation:

$$W_g = \frac{m_{dry}}{m_o \, C_o / 100} \tag{2.4}$$

where C_o is the initial polymer concentration.

2.7.3 Swelling Kinetics Measurements

For the swelling kinetics measurements, dry OVA gels were taken and immersed in distilled water (Figure 2.8). The weight changes of gels were measured gravimetrically at regular time intervals. The water uptake was calculated in terms of the relative mass parameter m_{rel}, given as:

$$m_{rel} = \frac{m_t}{m_{sw}} \tag{2.5}$$

where m_t is the mass of the gel sample at time t.

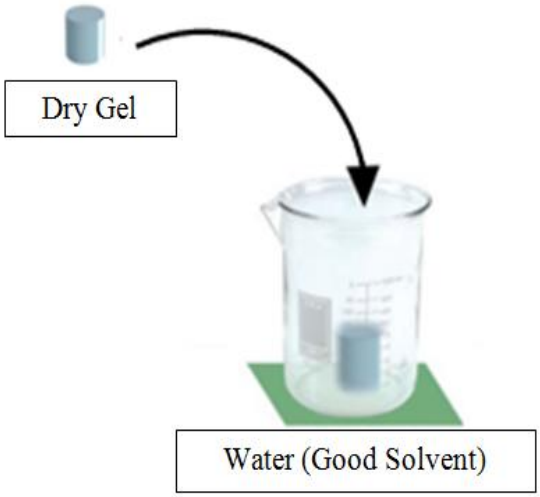


Figure 2. 7 Swelling of a dry gel in water

2.7.4 *In vitro* Cell Experiments

Before going through the cell experiments, the best cryogels were chosen regarding their mechanical and morphological properties.

2.7.4.1 Cell Adherence Test

A cell medium was prepared to culture the 10T1/2 cells. For this purpose, Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin was added in a cell culture flask at 37 °C and 5 % v/v of carbon dioxide (CO_2). As cell proliferation was enough for seeding the cryogels, the cell medium was removed and the cells were washed with PBS solution. Then after, 3-4 ml of trypsin was added to the flask containing the cells and incubated for 1 minute at 37 °C and 5 % v/v of CO_2 to detach the cells. Afterward, the cells were counted by Hemocytometer to calculate the cell number.

In order to prepare the scaffolds (5 w/v % OVA cryogels and OVA/HA biocomposites (with 1 w/v % HA) for cell culture studies, cryogels were cut into sections with 10 mm in diameters and 2 mm in thickness. And then, they were sterilized by 70% ethanol and incubated for 2 hours in culture medium at 37 °C and 5 % v/v of CO_2. For each scaffold section, $1x10^5$ cells/ml cells were seeded and incubated for 3 days. The culture medium was changed every day and evaluations were done at the 1^{st} and 3^{rd} days.

2.7.4.2 Live/Dead Staining Experiments

Live/Dead staining experiments were carried out to observe the cell viability. At first, culture media was removed and replaced with 500 µL of PBS. Then, cryogels were incubated at 37 °C for 10 minutes. Calcine (0.5 µL/1 ml) was added to PBS to dye the cells (staining), and then the solution was incubated for 30-45 minutes. Afterward, the cryogels were rinsed with PBS and left in the PBS solution.

2.7.4.3 Fluorescent Microscopy

After Live/Dead staining, the gels were examined under Fluorescent microscope. The photographs of the top and the bottom sides of each cryogel was taken at different magnifications.

2.7.5 Scanning Electron Microscopy Measurements

Scanning electron microscopy (SEM) was used to investigate the internal morphology of the dry gels. A Jeol JSM 6335F Field Emission Scanning Electron Microscope instrument was used for obtaining SEM images of the gel samples.

Figure 2. 8 Jeol JSM 6335F Field Emission Scanning Electron Microscope used for investigating the internal morphologies of dried gels

CHAPTER 3

RESULTS AND DISCUSSION

In this study, cryogelation was applied as a facile route to produce OVA and OVA/HA biocomposite scaffolds. In order to prepare these materials, crosslinking reactions were conducted in water at subzero temperatures either using a chemical crosslinker GA or urea to achieve physical crosslinking. Since the preliminary experiments showed that physically crosslinking is sufficient enough, further experiments were conducted using only urea instead of GA. Effects of some parameters, i.e, the initial polymer concentration and the crosslinker/HA amounts on the properties of the cryogels were investigated. As will be seen in the following paragraphs, both the obtained OVA cryogels and the OVA/HA biocomposite cryogels exhibited superior swelling and mechanical properties and were shown to be good candidates for TE applications.

3.1 The Gel Fraction Yield Calculations

Here, the effect of the initial polymer concentration on the efficiency of gelation process which is termed as the gel fraction yield was investigated. For this purpose, the initial polymer (OVA) concentration, C_0, was varied between 2 w/v % and 5 w/v %. The polymerization reactions were conducted at -12^{o}C for 24 hours. After 1 day of reaction, gravimetric calculations showed that insoluble polymer network was obtained even polymer concentration were very low such as 2 w/v %. This can be explained as for cryogelation systems, increasing the polymer or monomer concentration increase the polymer amount in the unfrozen micro-channels (cryo-concentration) and this increase enhances the crosslinking reactions and the concentration of the materials in these unfrozen regions [88].

Figure 3.1 shows the gel fraction of cryogels W_g versus the initial polymer concentration, C_0. As can be seen in the figure, it was found that 5 w/v % concentrated OVA cryogels showed the highest W_g value of 71% which is in accordance with the previously published values [161]. However, 2 w/v % concentrated OVA cryogels showed the lowest, 25 %, W_g value. Moreover, W_g was 46% for 3 w/v % and 57% for 4 w/v % concentrations. As can be clearly seen in Figure 3.1, the gel formation occurred successfully in every concentration values and it was increased by the addition of polymer content.

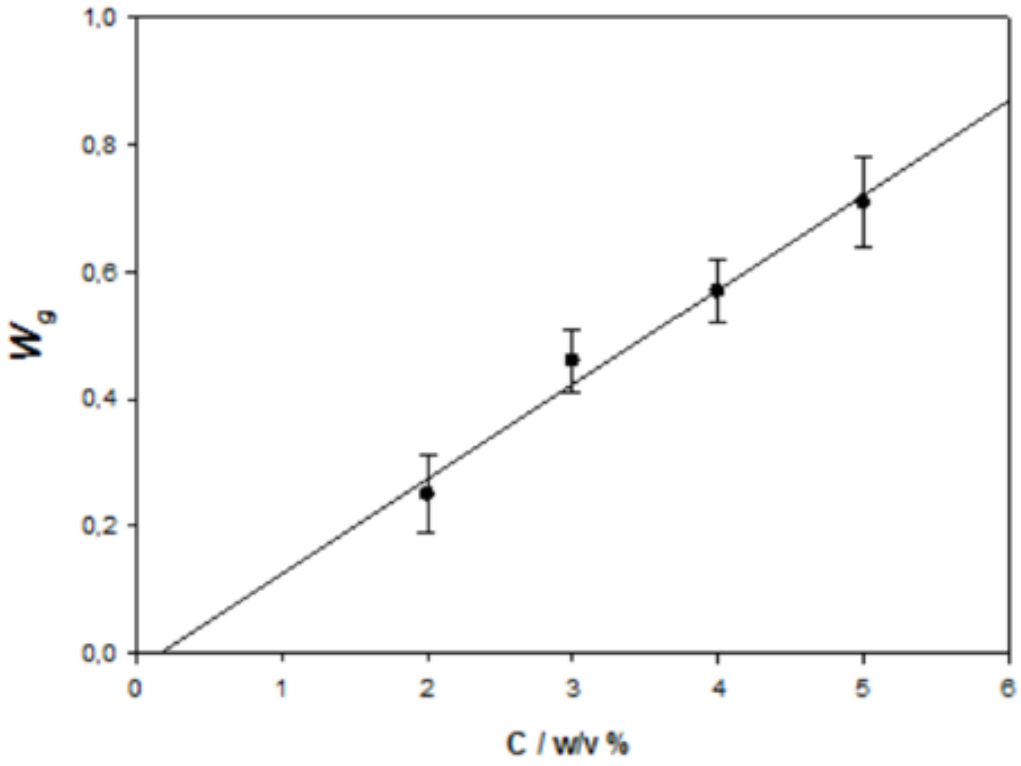


Figure 3. 1 The gel fraction, W_g of OVA cryogels as a function of polymer concentration

3.2 Scanning Electron Microscope

In order to visualize the porosity and morphology of both dried OVA and OVA/HA cryogels, SEM images were taken. Figure 3.2 shows the SEM images of the OVA gels with varying polymer concentrations between 2 w/v % and 5 w/v %. As can be seen in the Figure, all the cryogels exhibited excellent spongy morphology with highly interconnected three-dimensional internal structure.

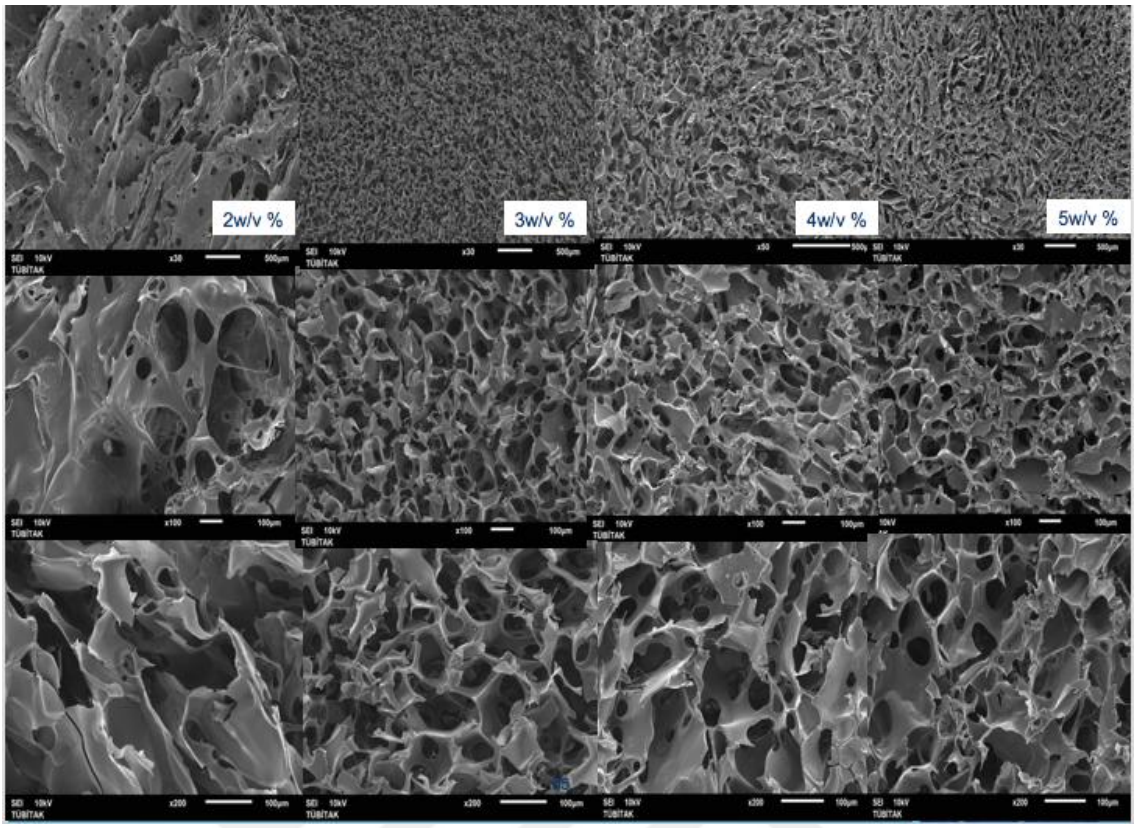


Figure 3. 2 The SEM images of OVA cryogels with varying concentrations between 2 w/v % and 5 w/v %

The average pore size of the cryogels was estimated from the images taken from the SEM. By measuring, at least, the diameters of 30 pores, we calculated the diameters as 55, 57 and 43 μm for 3, 4 and 5 % w/v respectively. As given in Table 3.1, the pore dimensions slightly decrease by increasing the polymer concentration. This result is a characteristic of cryogelation systems in which the frozen part consists of ice crystals while the unfrozen part contains the polymer and other additional molecules. Polymer molecules become denser in the unfrozen parts and by the increasing of the polymer concentration of the system, we allow more polymer deposition in the unfrozen part of the cryogel and hence we produce cryogels with thicker pore walls and narrower pore diameter.

As mentioned above, the natural bone structure consists of hydroxyapatite crystals. Therefore, we combined OVA cryogels with HA particles to obtain OVA/HA biocomposite cryogels. The polymer concentration was set to 5 w/v % and HA amount was varied between 0.0042 g and 0.1 g. Figure 3.3 shows the SEM images of the OVA/HA biocomposite cryogels with varying HA amounts. In addition, SEM images were also used to calculate the average pore diameter of OVA/HA biocomposite

cryogels with 5 w/v % polymer concentration and 0.042 g HA. The biocomposite cryogels showed a wide range of heterogeneous porous structure and the average pore diameter was calculated as 44 ± 15 µm.

Table 3. 1 Average pore diameter values as a result of varying OVA concentration

Polymer Concentration	**Average Pore Diameter**
3 w/v %	55 ± 16 µm
4 w/v %	57 ± 19 µm
5 w/v %	43 ± 16 µm

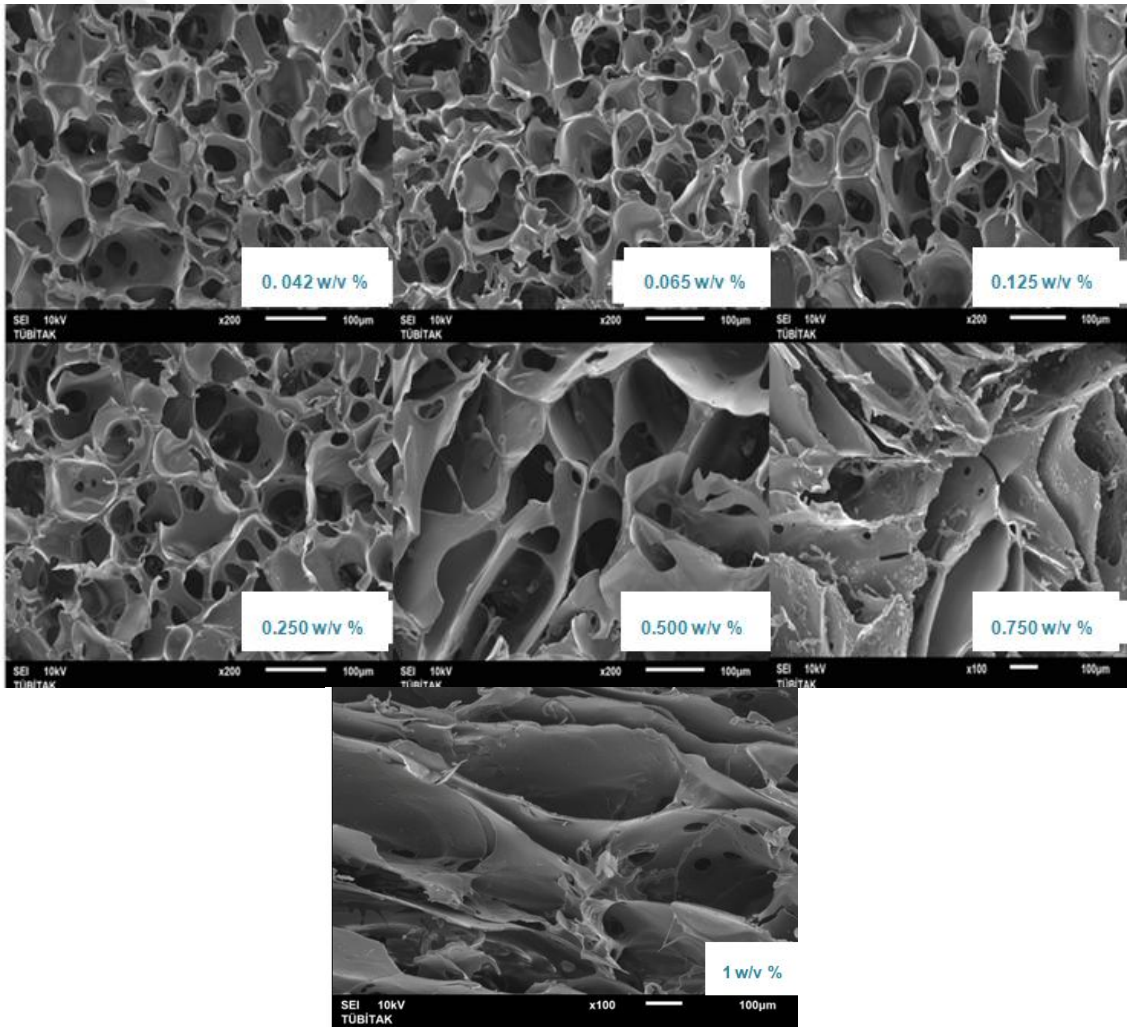
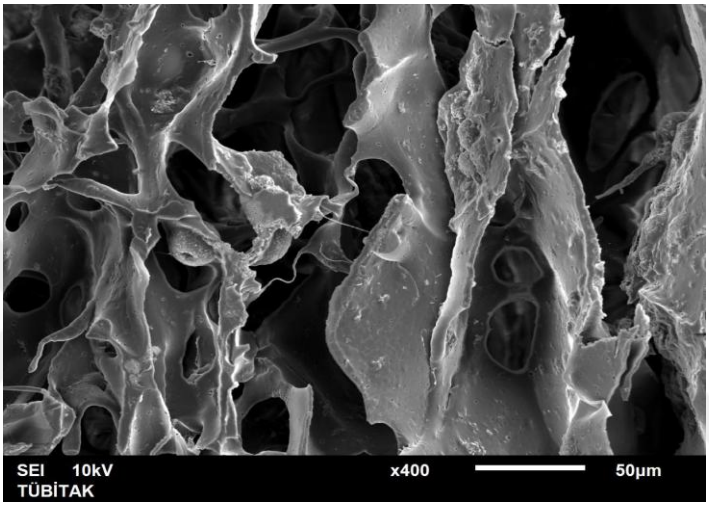


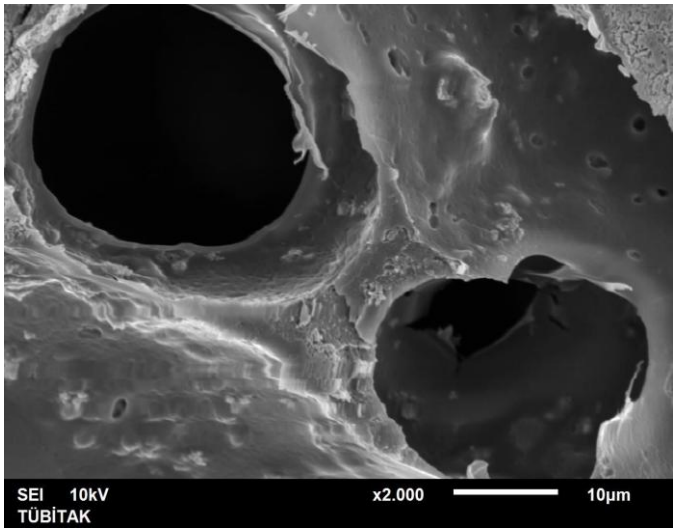
Figure 3. 3 OVA/HA biocomposite cryogels with varying HA concentration between 0.042 and 1 w/v %

Moreover, Figure 3.4 demonstrates the whole cross section surface of the OVA/HA cryogel with 5 w/v % OVA and 0.0125 g HA crystals. This figure proves that our biomaterial has highly interconnected porous structure. Therefore, the porosity measurements results showed that OVA/HA biocomposite cryogels are good candidates for bone tissue engineering applications.

Furthermore, the SEM images were taken at higher magnification in order to better visualize the HA crystals. In Figures 3.4 (A) and (B), HA clusters can be recognized on the pore walls of OVA cryogels with different resolutions which indicate the homogeneous dispersion of HA particles.



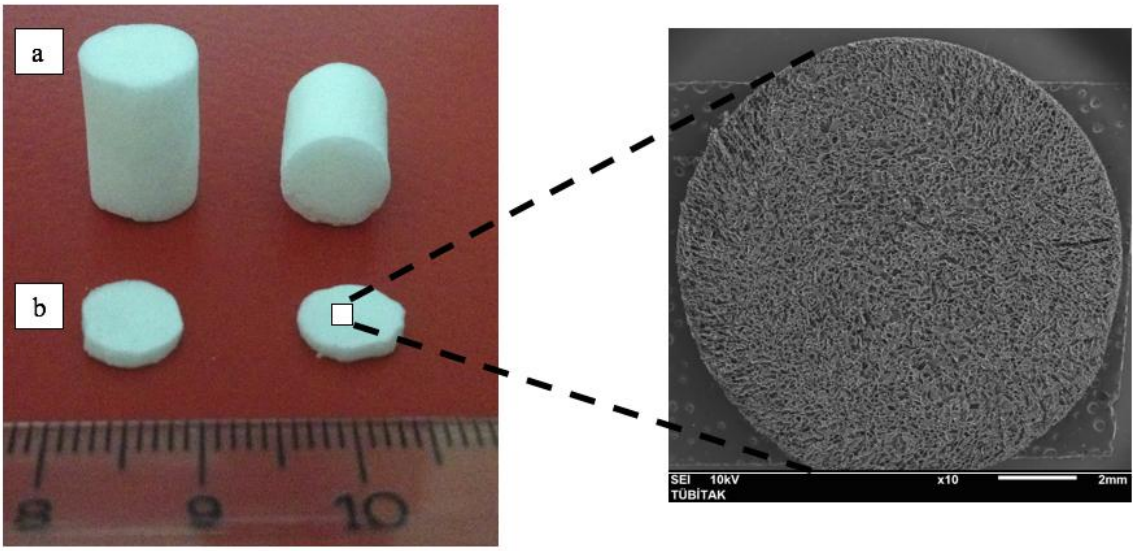
(A)



(B)

Figure 3. 4 The SEM images of 5 w/v % OVA cryogels with 1 w/v % HA at different magnifications (A) X400 (B) X2.000

Figure 3.5 also shows the cross-section of the dry cryogels which are in the cylindrical form. As obvious in the Figure 3.5 B, the SEM image of the cross-section of the cryogels exhibits a discontinuous surface.



(A) (B)

Figure 3. 5 The digital images of the dried monoliths (a) and disks (b) formats of ovalbumin cryogels with 5 w/v % OVA with 0.125 w/v % HA (A) and the SEM image of whole surface of cross section of OVA/HA cryogels with polymer concentration 5 w/v % with 0.125 w/v % HA (B)

3.3 Swelling Measurements

Another important feature of a scaffold in tissue engineering applications is the swelling characteristic that is water-binding and hydrophilic capability. So, swelling properties of the OVA and OVA/HA biocomposite cryogels were investigated in distilled water at room temperature. The equilibrium volume and weight ratios of the gels with respect to their both just after preparation and dry states were calculated as mentioned in Section 2.4.1.

When comparing the initial and the swollen states of a gel, the swollen gel is larger in size. Thus, a swollen gel has an expanded surface area which means larger surface/volume ratio, and cells can penetrate and adhere into the scaffold easily [198]. In other words, swelling enhances the infiltration of cells through the three-dimensional scaffold [198]. Moreover, the cell culture medium is absorbed by swollen gel; as a result nutrients can be efficiently transferred into the scaffold [198].

The weight and volume swelling ratios of the cryogels provide data for determining the internal structure of a scaffold [199]. When a porous gel is immersed in a solvent, the pores inside the network are filled with solvent. At the same time, the polymer part also absorbs solvent and the gel swells. There are two separate processes in the swelling of the porous gels: (1) solvation of network chains and (2) filling of the pores by the solvent [200]. While the equilibrium weight swelling ratio, q_w, includes the amount of solvent both in pores and in the polymer, the equilibrium volume swelling ratio, q_v, includes only the swelling of the network chains. Therefore, the high difference between q_w and q_v indicates that the sample contains a high amount of pores [200].

In this part of the study, the equilibrium swelling volume and weight ratios, q_v and q_w, of the OVA scaffolds with respect to the dry states are shown in Figure 3.6 as a function of polymer concentration. As can be seen in the Figure, increasing the polymer concentration leads to a dramatic decrease in q_w values and a slight decrease in q_v values. This decrease is due to the fact that increasing polymer concentration increases the polymer amount whereas decreases the porosity. Thus, the swelling capacity of a cryogel is decreased by increasing the polymer concentration. Moreover, one can easily notice the gap between q_w and q_v values for all the gels where the q_w values were nearly 30 times higher than q_v values. As reported before, the higher difference between q_w and q_v means the higher volume of the pores in the structure of the cryogels [200].

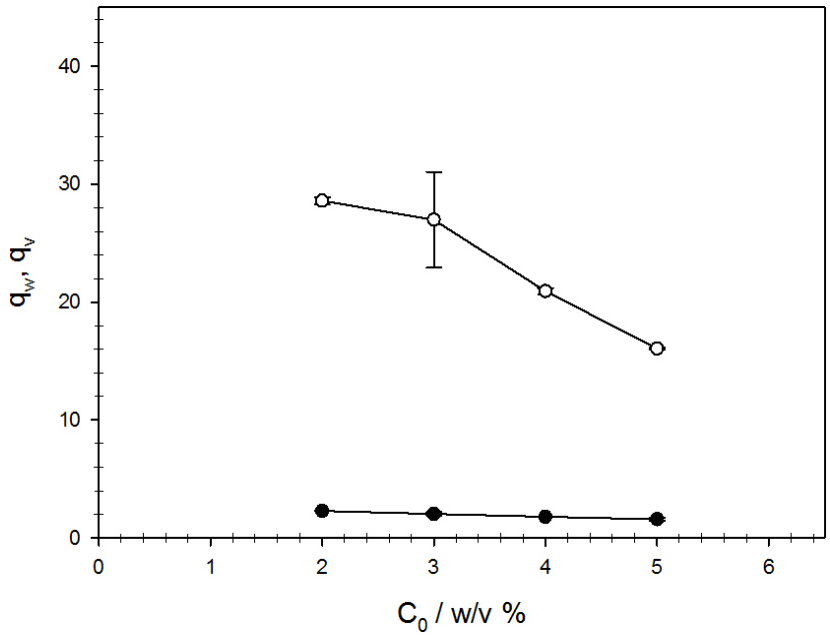


Figure 3. 6 The equilibrium weight, q_w and volume, q_v ratios of the OVA cryogels with respect to their dry states by changing polymer concentration (Open and close symbols stand for q_w and q_v, respectively.)

The gap between equilibrium weight and volume swelling ratios, q_w and q_v were also observed for the hydroxyapatite incorporated samples. As shown in Figure 3.7, while increasing the HA amount results in the decrease in q_w values, the q_v values did not change significantly. Such a decrease in q_w values was reported and explained previously as the hydroxyapatite molecules are embedded and accumulated into the pore walls of the cryogel [3]. Furthermore, the hydrophobic characteristic of hydroxyapatite molecules also contributes to the decrease in swelling capacities of the samples [3].

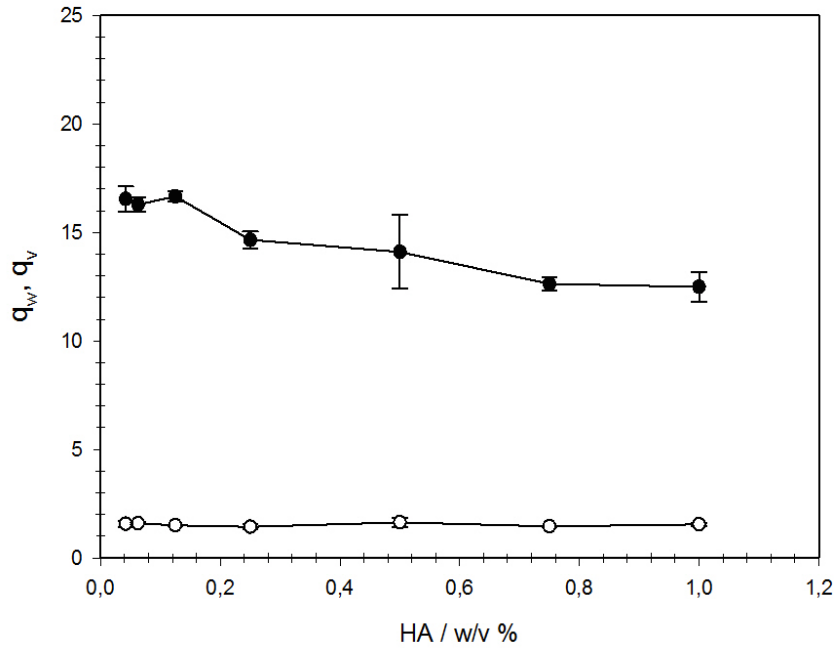


Figure 3. 7 The equilibrium weight, q_w and volume, q_v ratios of the OVA cryogels with respect to their dry states by changing HA concentration

3.4 Swelling Kinetics

The swelling kinetics measurements of the samples were carried out gravimetrically at room temperature. OVA cryogels with various hydroxyapatite amounts were subjected to swelling process in water. The results are shown in Figures 3.8 and 3.9 where the relative gel mass, m_{rel} is plotted against swelling time. Here, dry gels were immersed in distilled water and weight changes were recorded at certain time intervals. As can be seen in Figures 3.8 and 3.9, the gels swelled within 10 sec and reached equilibrium in a few minutes. The superfast swelling rate of the cryogels is also attributed to the connectivity of the pores which plays a vital role in fast swelling; water can enter the gels through convection which allows faster transportation of the water through the

cryogels. Moreover, this rapid swelling has an advantage in tissue engineering applications as it contributes to the fast absorption of the cell culture medium.

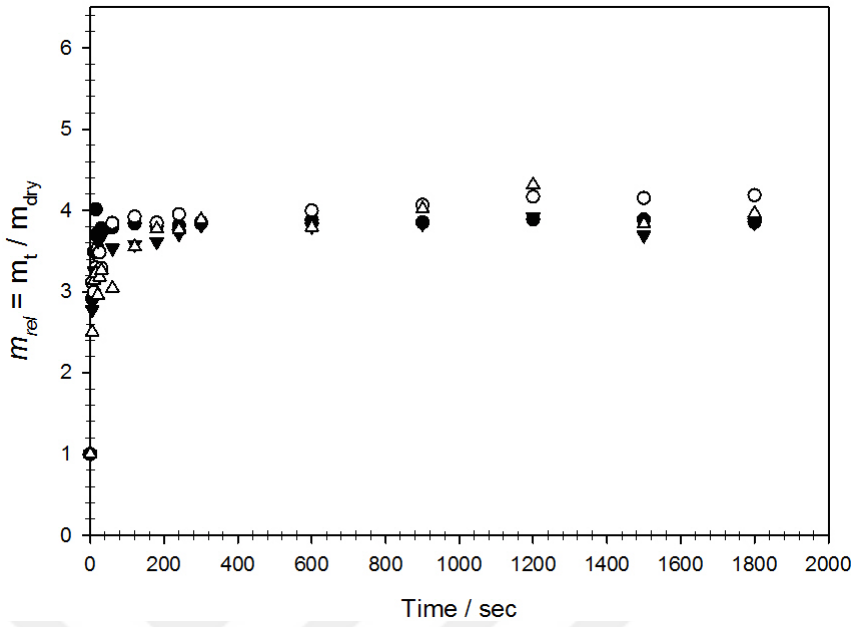


Figure 3. 8 Swelling kinetics of OVA cryogels (C_0 = 2-5 w/v %)

Moreover, in order to investigate the effect of hydroxyapatite molecules on the swelling rate of the cryogels, a comparison has been made between with and without HA incorporation. As can be seen in Figure 3.9, HA has an insignificant effect on the swelling rate of the cryogels.

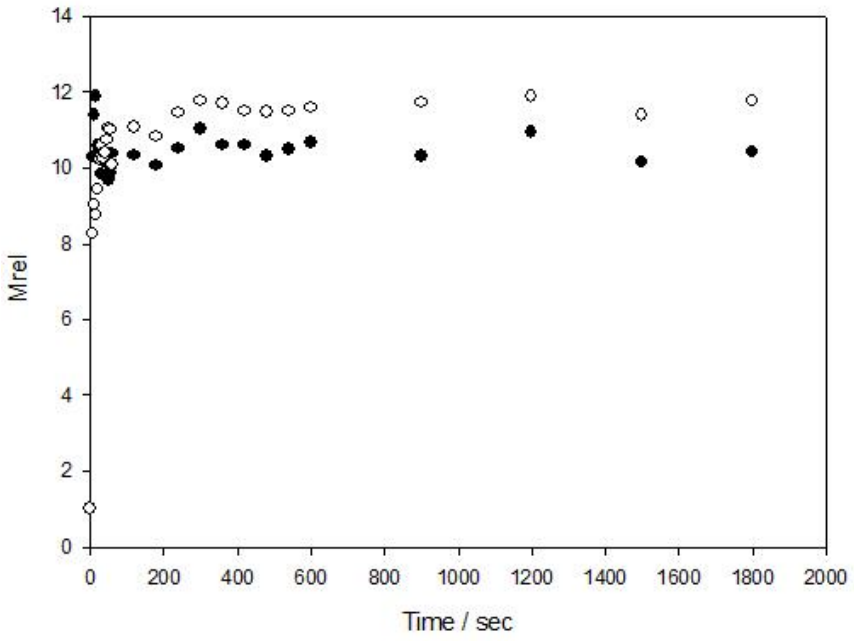


Figure 3. 9 Swelling kinetics of OVA cryogel and OVA/HA biocomposite cryogel with 1 w/v % HA

3.5 Mechanical Properties of the Biocomposite Cryogels

In order to investigate the mechanical properties of the cryogels, the samples were squeezed between the fingers. As shown in Figure 3.10, the cryogels were very tough; no mechanical destruction was observed upon removal of the force. The gels could withstand mechanical stress without any crack formation and deformation. Once the forces were no longer applied, the samples returned to their original shapes. This superior elasticity of the cryogels is due to the large pores surrounded by dense pore walls.

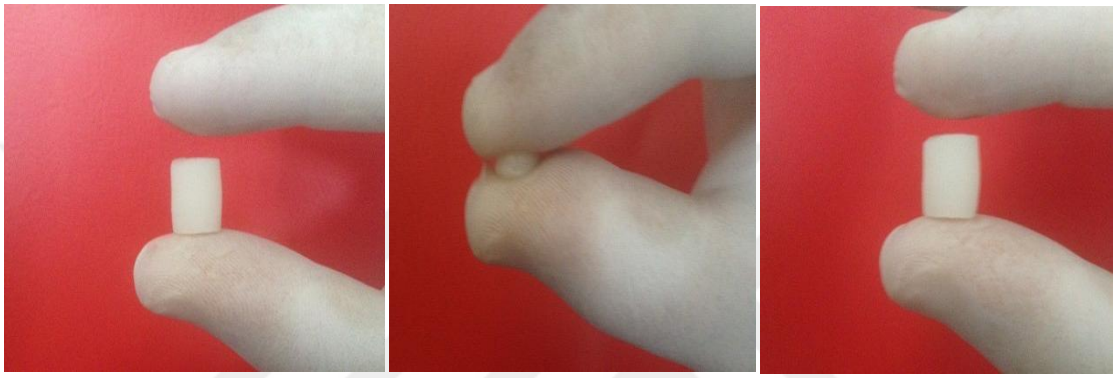
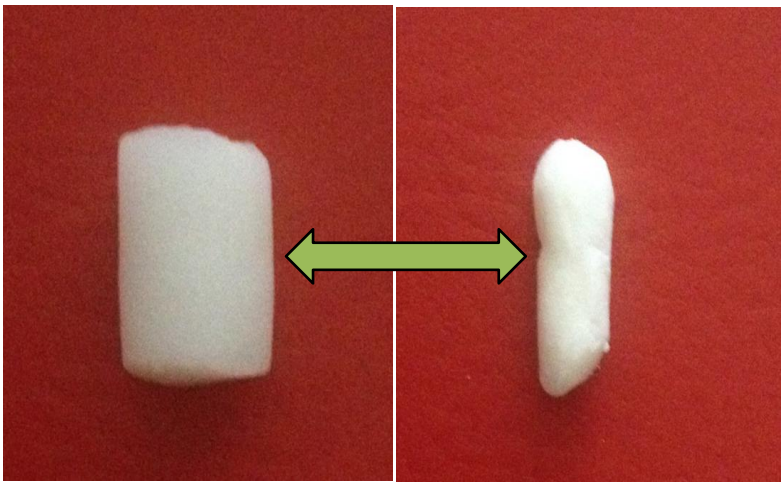


Figure 3. 10 The digital images swollen 5 w/v % OVA with 1 w/v % HA biocomposite cryogels when being squeezed between fingers

Figure 3.11 shows the water swollen and squeezed states of the cryogel with 5 % w/v OVA and 1 w/v % HA. This Figure also indicates that the cryogels excellently retain water and takes its original shape without any cracks upon squeezing.



(A) (B)

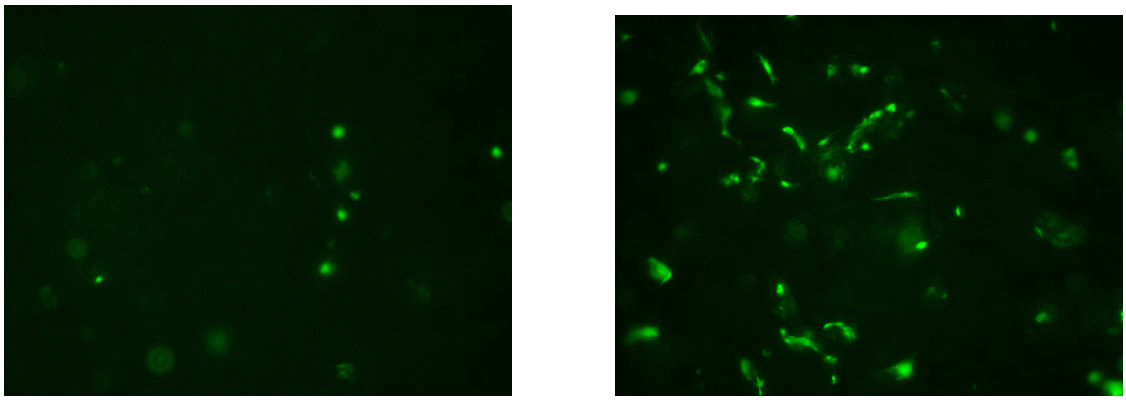
Figure 3. 11 The digital image of the swollen cryogel (A) and the squeezed cryogel (B) with 5 w/v % OVA and 1 w/v % HA

3.6 In vitro Cell Viability, Proliferation and Biocompatibility Experiments

As presented in the previous sections, porosity, and mechanical characterization results have shown that OVA cryogels and OVA/HA biocomposite cryogels are good candidates for tissue engineering applications since they have large interconnected porous structures and they are durable under stress. In order to investigate the availability of the cryogel as biomaterials for tissue engineering applications, they were combined *in vitro* with mouse embryonic fibroblast cells which are called as the 10T1/2 cell line. In terms of the cell viability and proliferation, live staining experiments were run to stain alive cells. The staining procedure was performed as explained in Section 2.7.4.

Based on the mechanical properties and internal morphology results we picked 5 w/v % OVA and 5 w/v % OVA with 1 w/v % HA for cell experiments. Each type of cryogel was prepared for cell culture procedure as mentioned in Section 2.7.4 and then seeded with 10^6 cells/ml 10T1/2 cells.

In order to demonstrate the cell-biomaterial interaction during cell viability experiments, seeded cryogels were examined under the fluorescent microscope for 3 days. The results of the first day are presented in Figure 3.12 where the green parts of the images indicate alive cells. As one can easily recognize, the cell number was very low in Figures 3.12 (A). However, in Figure 3.12 (B), there are many alive cells with spindle shape and are seemed as interacting to one another.

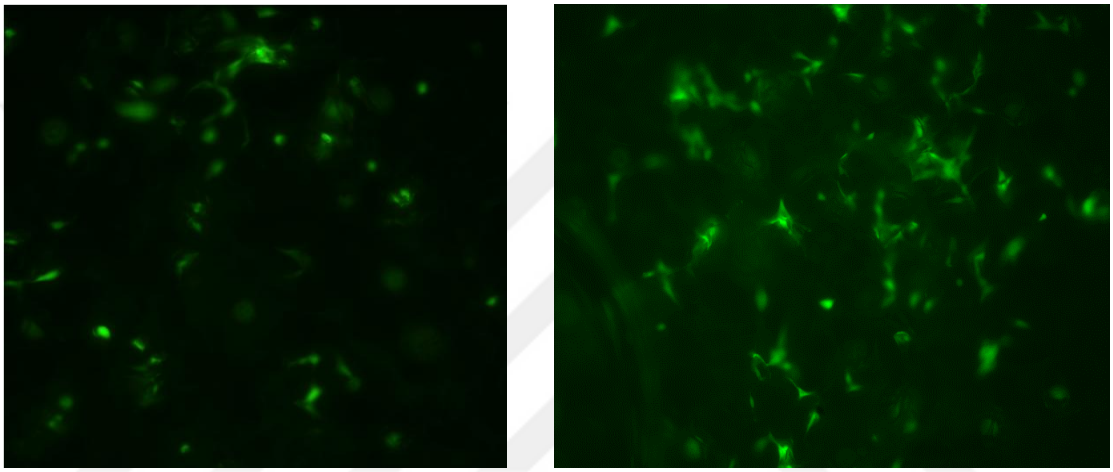


(A) (B)

Figure 3. 12 Fluorescent microscopy images of 5 w/v % OVA cryogel (A) and 5 w/v % OVA with 1 w/v % of HA (B) at day 1.

As mentioned above, the interaction of 10T1/2 cells and cryogels was examined at day 1 as well as at day 3. At day 3, the trend of the alive cells was significantly changed.

The Figure 3.13 corresponds to the results of day 3. As it is evident in this figure, the cell number and cell to cell interaction were obviously increased at day 3 on both cryogels (Figures 3.13 (A) and (B)). Especially, a significant rise was observed for OVA with 1 w/v % HA biocomposite cryogels which are obvious in Figure 3.13 (B). There are many flattened alive cells in excellent dorsal shape and clustered cell layers which are seemed to be secreting ECM. The combining of HA molecules into the cryogel network enhanced the cell adhesion, proliferation and cell layer formation. As proven previously [15], the incorporation of HA molecules within the cryogel structure improves the interaction of the cell and cryogel as observed the boost in cell number.



(A) (B)

Figure 3. 13 Fluorescent microscopy images of 5 w/v % OVA cryogel (A) and 5 w/v % OVA with 1 w/v % of HA (B) at day 3.

CHAPTER 4

CONCLUSIONS AND FUTURE WORK

4.1 Conclusions

The general aim of this thesis was to produce protein-based biomaterials for TE applications using the cryogelation method. A series of OVA cryogels were prepared with (OVA/HA) and without HA (OVA) by dissolving OVA in aqueous urea solutions and then keeping these solutions at -12^{o}C for 24 h. The effects of the polymer concentration and HA amount on the properties of the cryogels were investigated. The conclusions that can be drawn from the results of the study are as follows:

- Cryogelation reactions of OVA and OVA/HA at subzero temperatures led to the formation of interconnected macroporous structures. The SEM measurements revealed that the obtained scaffolds have highly porous internal morphologies.

- OVA cryogels were able to be prepared at even low polymer concentration such as 2 w/v % due to the cryoconcentration effect.

- Urea was successfully used as a crosslinker in order to produce OVA cryogels and OVA/HA biocomposite cryogels without any need for hazardous chemical crosslinkers.

- The yield of gel fraction calculations proved that increasing the polymer concentration enhances the gel formation. It was shown that the cryogels with the concentration of 5 w/v % had the highest gel fraction percentages.

- The effect of polymer concentration on the internal structures of cryogels was also investigated. SEM measurements showed all produced cryogels have

excellent macroporous sponge-like structures. The pore diameter was slightly decreased by increasing the polymer concentration.

- In order to make the OVA cryogels good candidates for bone TE, HA was incorporated to the reaction formulation. A HA clusters visualization was achieved on the inner walls of the samples for the cryogels with OVA/HA (1 w/v %).

- The swelling characterization of the cryogels exhibited that the prepared cryogels have superfast swelling behaviors; the gels absorbed the water in just 10 sec. The equilibrium swelling of the gels showed that the dry samples could swell 10-30 folds of their drying weights. Moreover, the difference between equilibrium weight and volume swelling ratios revealed that both OVA cryogels and OVA/HA cryogels are consisting of highly porous internal structures. The effect of HA on the swelling behavior of the cryogels was also highlighted. The results showed that OVA cryogels without HA addition showed slightly better swelling rates.

- The prepared cryogels were mechanically durable, compressing the samples between fingers did not cause any crack development. The cryogels presented crack-free structures under force and after the force was removed they could retain their initial shapes.

- 5 w/v % OVA cryogel and 5 w/v % OVA with 1 w/v % HA biocomposite cryogel were chosen for *in vitro* cell experiments. In order to investigate the biocompatibility of our scaffolds, cell viability assay was followed for 10T1/2 cells. The alive cells represented a green color with the aid of calcein dye. The fluorescence microscopy outcomes showed that the addition of HA crystals enhanced cell adhesion and proliferation on the cryogels. At the end of the day 3 of cell culture, there were many alive cell colonies which were in contact with each other and pretending to migrate to the inner parts of the OVA/HA biocomposite cryogel.

- In conclusion, gel fraction, swelling, mechanical, porosity and cell studies results proved that the newly synthesized OVA/HA biocomposite cryogels are

potentially novel candidates as scaffolds for bone tissue engineering applications.

4.2 Future Work

This study was carried out to characterize newly produced OVA/HA biocomposite cryogels. However, we were not able to fully characterize the scaffolds. As further studies, XRD and FTIR characterization procedures can be included for better understanding the structures.

Moreover, 10T1/2 mouse embryonic fibroblast cells were combined *in vitro* studies to observe the biocompatibility of the cryogels. For the following studies, an osteoblastic cell line such as MG63 osteoblast-like cells can be investigated in terms of the osteogenic differentiation. Furthermore, several important procedures such as MTT and DNA extraction assays, alkaline phosphatase activity and histological analysis can be carried out. In addition, *in vivo* studies can be explored in order to get more clear idea about the immunological responses of the living organisms to the biocomposite cryogels.

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CURRICULUM VITAE

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EDUCATION

Degree	Department	School/University	Date of Graduation
Undergraduate	Biology	Fatih University	07/08/2012
High School	Science and Mathematics	Beyoglu Anatolian High School	15/06/2007

SCHOLARSHIPS

1. Republic of Turkey, Ministry of National Education, Ph.D. Scholar, 2015-

2. Katholieke Universiteit Leuven, Belgium-Yildiz Technical University, Turkey, Erasmus Student Exchange Program, 2013-2014.

WORK EXPERIENCE

Year	Corporation/Institute	Enrollment
2015	VGS Organization	Editor
2015	Burc Genetics Diagnostic Center	Molecular Biologist

2012	Republic of Turkey Ministry of Justice	Intern
2011	Istanbul University School of Medicine	Intern
2010	Burc Genetics Diagnostic Center	Intern

PUBLISHMENTS

Conference Papers

1. **Akkaya, K. B.,** Ozmen, M. M., Patterson, J., "Ovalbumin Biocomposite Cryogels as Tissue Engineering Scaffolds", 11th International Symposium on Frontiers in Biomedical Polymers Riva del Garda, Italy, July 8-11, 2015.

PROJECTS

1. "Production of Protein-Based Macroporous Tissue Scaffolds from Natural Polymers/ Protein Bazlı Doğal Polimerlerden Makrogözenekli Doku İskelesi Eldesi" Research Assistant, Yıldız Technical University, YULAP, Grant No: 2014-07-04-YL03.