REPUBLIC OF TURKEY
ACIBADEM MEHMET ALİ AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

DESIGN AND CHARACTERIZATION OF HYDROGEL MODELS CONTAINING NK-92 OR HEK293T CELLS FOR SKIN TISSUE ENGINEERING

SİBEL CENDERE

MASTER THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

SUPERVISOR
Assoc. Prof. Beste Kınıkoğlu Erol

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DECLARATION

I hereby declare that; this thesis has been written by me based on the data obtained in line with the scientific rules and ethical principles of responsible conduct of research. All information, data, comments, analysis have been collected and processed through scientific, academic writing style, and literature used have been duly shown by giving reference to the original sources in accordance with the publication ethics. I also announce and emphasize that I have not violated any rules secured by patent and copyrights whilst the conduct and writing of this research.

22.06.2021
Sibel Cendere

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LIST OF ABBREVIATIONS AND SYMBOLS

ALG : Alginate
ASC : Adult stem cell
$CaCl_2$: Calcium chloride
Conc. : Concentration
DMEM : Dulbecco's Modified Eagle's Medium
DMSO : Dimethyl sulfoxide
DW : Distilled water
ECM : Extracellular matrix
EDTA : Ethylenediaminetetraacetic acid
ESC : Embryonic stem cell
FBS : Fetal Bovine Serum
FT-IR : Fouirer Transform Infrared
GA : Glutaraldehyde
GAG : Glycosaminoglycan
GEL : Gelatin
GF : Growth factor
HA : Hyaluronic acid
HAP : Hydroxyapatite
hASC : Human adipose-derived stem cell
HEK : Human Embryonic Kidney
IL : Interleukin
iPSC : Induced pluripotent stem cell
LV : Low vacuum
MBG : Mesoporous bioactive glasses
µg : Microgram
µL : Microliter
µm : Micrometer
mL : Milliliter
mM : Millimolar

mins	**:** Minutes
MSCs	**:** Mesenchymal stem cells
MW	**:** Molecular weight
NK	**:** Natural Killer
PBS	**:** Phosphate Buffer Saline with Calcium and Magnesium
PBS w/o	**:** Phosphate Buffered Saline without Calcium and Magnesium
Pen-Strep	**:** Penicillin-Streptomycin
PLGA	**:** Poly(lactic-co-glycolic acid)
rpm	**:** Revolutions per minute
RT	**:** Room temperature
SEM	**:** Scanning electron microscope
SHH	**:** Superabsorbent hybrid hydrogel
vol.	**:** Volume

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SUMMARY

Skin Tissue Engineering, a sub-branch of Regenerative Medicine, works on systems for repairing or regenerating damaged skin tissue. Hydrogels, one of the techniques developed for these purposes, provide extracellular matrix (ECM) secretion and trigger intercellular signaling pathways, thus inducing cell adhesion, proliferation and differentiation depending on the materials selected in the design. The aim of this thesis is that this research, which was developed for defective skin tissue, could be a prospect for melanoma treatment. Consequently, different from cell-hydrogel practices in the literature, NK-92 cells, which has anti-tumor activity and is frequently used in various cancer therapies, was selected. In the second step, suspension NK-92 cells were seeded into these distinct hydrogels. Subsequently, viability percentages of cells were analyzed. Since this research has not been found before in the literature, while investigating the cooperation of suspension cells with hydrogel, in order to demonstrate shortages and differences, adhesive HEK293T cells were seeded into the identical hydrogels. As a result of cell seeding practices, it was observed that hydrogels with the desired properties lost some of their properties when combining with cells and cell culture medium. It was observed that the deformation level increased and the stiffness rate declined with the effect of the decrease in the degradation time. As a result of these changes, cells are deprived of the crucial environment for survival and proliferation. To enhance these hydrogels, the ratio of biopolymers and crosslinker used, or type of one or more of the biopolymers, can be varied.

Keywords: Artificial Skin Layer, Hybrid Biomaterials, Interpenetrating Hydrogels, 3D Disease Models, 3D Networks

ÖZET

Deri Doku Mühendisliği İçin NK-92 ya da HEK293T Hücreleri İçeren Hidrojel Modellerinin Tasarımı ve Karakterizasyonu

Rejeneratif Tıp alanının bir alt dalı olan Deri Doku Mühendisliği, hasar görmüş deri dokusunu iyileştirmeye ya da yenilemeye yönelik sistemler üzerinde çalışmaktadır. Bu amaçlarla geliştirilen tekniklerden biri olan hidrojeller, tasarımında seçilen organik/sentetik materyallere göre; hücre dışı (ekstrasellüler) matriks salgılanmasını, böylece hücrelerarası sinyal yolaklarını tetikleyerek hücre adhezyonunu, hücrelerin çoğalmasını ve farklılaşmasını sağlamaktadır. Bu tezin amacı, literatürde bulunan hücre-hidrojel kombinasyonlarından farklı olarak, anti-tümör aktivitesi bulunan ve çeşitli kanser terapilerinde öncü olarak kullanılan NK-92 hücrelerinin kullanılmasıyla, defektif deri dokusuna yönelik geliştirilen bu çalışmanın özellikle melanoma için bir tedavi umudu olabileceğidir. Çalışmanın ilk basamağında, hücre yapısına uygun olarak seçilen hyalüronik asit, jelatin ve aljinat biyopolimerleri kullanılarak tasarlanan porlu yapıdaki hidrojellerin optimizasyonu yapıldı. İkinci basamakta bu hidrojellere, optimizasyonu sağlanmış süspanse halde bulunan NK-92 hücrelerinin ekimi yapılarak, hayatta kalma yüzdeleri test edildi. Literatürde bu çalışmaya daha önce rastlanmadığından, süspanse hücrelerin hidrojel ile birlikteliği test edilirken eksiklikleri tespit edebilmek için kontrol amaçlı aynı hidrojellere optimizasyonu sağlanmış yapışkan HEK293T hücreleri ekildi. Hücre kültür çalışmaları sonucunda; istenilen özelliklere sahip hidrojellerin, hücre kültürü ile birleşmesiyle bazı özelliklerini kaybettiği gözlendi. Degredasyon süresinin düşmesiyle deformasyon seviyesinin arttığı ve katılık oranının düştüğü gözlendi. Bu değişimler sonucunda, hücreler hayatta kalabilmek ve çoğalabilmek için gerekli ortamdan mahrum kalmaktadırlar. Hidrojel modelinin iyileştirilmesi için; kullanılan biyopolimer ve çapraz bağlayıcı oranları ya da biyopolimerlerden biri veya fazlası değiştirilebilir.

Anahtar Sözcükler: Hibrit Biyomalzemeler, İç İçe Geçen Hidrojeller, Üç Boyutlu Ağ Yapıları, Üç Boyutlu Hastalık Modelleri, Yapay Deri Tabakası

1. BACKGROUND AND AIM OF STUDY

Tissue engineering is a sub-branch of regenerative medicine that aims to produce artificial tissues with biocompatible, biodegradable, non-immunogenic and non-toxic elements to replace damaged or lost tissues. Subsequently, this 'artificial tissue' transplanted into the patient involves seeding specific cells on structures and providing an environment for the secretion of ECM components called "scaffolds". Scaffolds should be able to mimic natural tissue and provide an environment for cells to perform their vital functions (3).

Scaffolds, since they are capable of simulating the natural environment of living tissue, could be particularly effective to 3D *in vitro* cell culture applications. Cell cultures developed in 3D display more authentic performance than monolayer cell applications. Forcing the cells to attach to a flat and stable place seriously affects their metabolism and decreases in vital functions (26). Therefore, the effects of cell interactions and the external environment on the improvement of cells can be examined. The aim of *in vitro* cell culture studies is to adapt *in vivo* conditions as accurate as possible. There are specific requirements for each cell line used, thereof the optimization should not be done under the same conditions for each cell type. Conditions provided in particular situations should present the most reliable results (4).

In this thesis, hydrogel was utilized as scaffold and hydrogel design procedures were developed based on the hydrogel structure defined in the article "*Development of UV crosslinked biodegradable hydrogel containing adipose derived stem cells to promote vascularization for skin wounds and tissue engineering*" (22). The hydrogel constructed in this thesis consists of the combination of alginate (ALG), hyaluronic acid (HA) and gelatin (GEL) polymers crosslinked with calcium chloride ($CaCl_2$). The

preferred polymers are naturally obtained polymers that are well-known to cells in their natural environment and can provide living conditions close to natural tissue.

The use of hyaluronic acid increases the stability of the hydrogel and affects the pore size in accordance with its concentration. It allows the cells to colonize into the pores, to proliferate by supporting their vitality, and to transfer the nutrient components (23). In addition, hyaluronic acid has a significant role in augmentation and regeneration, as it regulates ECM and provides wound healing (24). Alginate-gelatin composition supported by Ca^{+2} enhances cell adhesion and proliferation. Since gelatin has positively charged residues, such as arginine and lysin, it enables the growth factors (GFs) and ECM components to function collectively (25). The use of alginate, on the other hand, is liked by the cells, as it is a biocompatible material and promotes the gelation process. Furthermore, it is easy to access and preferred by reason of it is low-cost (26).

This hydrogel design has been developed as a hope for injured or diseased skin tissue, specifically burns and skin cancers. For this purpose, NK-92 cell line was used as a cell type that was not found to be applied together with hydrogels in literature. NK-92 is frequently used as an immunotherapeutic agent, exclusively in cancer therapies. It is a highly preferred cell line with its features of being easily reproduced in cell culture, long-term storage conditions, can be used as allogenic and not leading to graft-versus-host disease. Particularly high activity against solid tumors such as melanoma has been shown in several studies (27). In line with this information, it is intended to establish a cellular cancer therapy under the guidance of tissue engineering, which is considered being hope for future studies.

However, since NK-92 is a suspension cell type and there are no such investigations in the literature, there is no report about the ability of suspension cells to colonize and maintain their functions in hydrogel scaffolds. In order to understand

the requirements and shortcomings during optimization, HEK293T cells, which are adherent and easy to study in cell culture, were simultaneously seeded in the same designed hydrogel formations and the results were evaluated comparatively.

The aim of this study is to pave the way for the use of hydrogel preparations, which are used in various skin injuries and are still being developed, in skin cancer treatments.

2. INTRODUCTION

2.1. Tissue Engineering

The term ‘tissue engineering’ was defined by Langer and Vacanti for the first time in 1993 as follows: “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 function” (1). In the past 30 years since the early studies were made, tissue engineering, along with the rapidly progressing technology, has turned into today’s reality, which was a conception of that time. Although studies on many tissues and organs are developed in their simplest but valuable design, the same strategies are still used today because they provide a remarkably substantial basis.

In recent years, despite the technological advances in medicine, thousands of individuals pass away every year as a result of cannot be found an organ donor or another suitable substitute for their damaged or diseased organs. Tissue engineering as a sub-branch of regenerative medicine, is developed with the help of biological tools such as biomaterials which is commonly represented as materials used in medical devices, and growth factors (GFs), provides a therapeutic solution to challenging medical conditions based on the principle of replacing damaged or diseased tissues and organs *in vivo* with artificially constructed *in vitro* tissues and organs. Tissue engineering works interdisciplinary with cell biology, material science, engineering, chemistry, molecular biology, medicine and many other fields.

Tissue engineering applications are divided into two groups as therapeutic and diagnostic. In therapeutic applications, artificial tissue is produced and transplanted to the patient, or it is grown in the patient’s body by particular instruments. In diagnostic

applications, artificial tissues produced *in vitro* are used in various drug screening tests (35).

Some tissue and organ regeneration capacity is very low and makes it challenging for clinicians to develop treatment in such situations. The risk of tissue rejection is extremely high in tissue and organ transplantation, which is the method generally used in such cases. In order to eliminate this risk, the mechanical devices developed for lost or injured tissues are very limited in their ability to perform the characteristics of native tissues and to control the condition of the patient effectively. In addition, the need for *in vitro* studies has arisen due to the fact that animal models produced for human testing do not perfectly reflect the responses occurring in the human body, are also costly and lead to ethical issues (2). Tissue engineering, which emerged as a result of these complications, is remarkably successful and promising in terms of its capacity to reflect *in vivo* systems and improve biological functions. It provides the opportunity to recover damaged or diseased tissues and organs or to reconstruct them *in vitro* by using cells with numerous engineering applications together with appropriate biochemical and physiological factors. Briefly, it is a developing technological discipline concerned with the processes of development and growth through regenerative medicine.

The main purpose of tissue engineering applications is to repair damaged tissue or replace it with a developed artificial tissue through cell transplantation. Artificial ECM biomaterials, which surround the cells, provide space and mechanical support for new tissue formations, are a critical component in these new tissues produced. The composition and morphology of the ECM consists mainly of water, fibrous proteins (such as collagen, elastin, fibronectin, and laminin) and proteoglycans (such as hyaluronic acid, perlecan, decorin) depending on the tissue and cell population in which it is located (48). ECM obtained with various biomaterials generates a suitable environment both physically and chemically by performing cell and organ interactive

functions as well as the secretion of new ECM and presenting ligands that specifically bind to cell receptors (3).

Tissue engineering has a potential for the reconstruction of congenitally damaged organs, as well as organs damaged by trauma, age, and neoplasm (3). Therefore, the improvement of bioactive scaffolds, progenitor cell biology, cell-cell interactions and interactions with the ECM, the biomechanics, morphology and physicochemical structure of *in vivo* systems, effects of ECM on cellular activities, study on compatible animal models, the effects of GFs and GFs combinations on cells are discussed. Although these studies have shaped our view on *in vitro* tissue cultures, cell biology, and artificial tissue and organ development, technological studies focus on techniques aimed at regeneration of damaged or deformed tissues *in situ*.

Tissue engineering approaches mainly consist of cells, scaffolds and GFs and its processes begin with harvesting cells from the patient or another individual (28). The cells can be stem cells, immature cells, or cells that can already present the characteristics of organs, often to be utilized as a mixture of various cell types (35).

Implantation products are designed to achieve their functions at the time of application or after implantation. This process, which is also defined as ex vivo tissue engineering, includes the expansion and differentiation of cells or the modification of progenitor cells in culture (1). These fundamental processes support the proliferation and differentiation of cells or progenitor cells in the functional tissue where the produced biomaterial is implanted with GFs and to form the basis for cellular functions such as cell-cell interactions, production of biomolecules and ECM formation. When performing a new artificial tissue, essentially three procedures are applied:

- Isolation of only required cells and substitutes to be utilized in the system to reduce the risk of immune rejection.
- Achieving tissue or tissue-like structures by various techniques. In order for this application to develop as desired, sufficient signaling molecules must be produced on a large scale.
- Production of 3D cell culture systems by seeding the isolated cells into the formed matrix structures.

In artificial tissue design, there are crucial factors to consider, specifically regarding cell source, such as not causing ethical issues depending on the origin of the cell type chosen, mechanical properties of the cell, cell culture conditions, scaffold construction, well-optimized storage conditions, and establishment of specialized cell banks for such cell groups, thus, they can be cultured repeatedly on a large scale when needed.

2.2. Skin Tissue Engineering

Skin is a connective tissue with an extremely high self-renewal capacity, consisting of ECM, fibroblasts, vascular endothelial cells, sweat glands, sebaceous glands, hair follicles, blood vessels and nerve endings. As the largest organ in the body, it has a significant role in performing vital functions. Essentially, it protects the body against external impacts and microorganisms, keeps the metabolism under control together with electrolytes, body fluids and nutritional components, and plays an effective role in thermoregulation. It gains mechanical strength and resilience with collagen and elastin secreted by fibroblasts (8).

Many people struggle with a variety of skin diseases, such as skin ulcers or second- and third-degree burns, and demand for new tissue to replace with their injured

or diseased tissue. Even before the emergence of modern medicine, health professionals developed various approaches to treat skin injuries or to protect wounds from external influences. For these purposes, autologous skin grafting (skin autografts), skin allografts, xenografts and amnion techniques have traditionally been applied.

Skin autografting involves the transplantation of tissue obtained from the patient's own body for epithelial tissue that has lost its ability to regenerate. There is no risk of tissue rejection in autografting, which is a widely used practice in India, specifically in premodern times (11).

In allografting, tissue obtained from another individual's body is transplanted into the patient's body. Clinical applications of the human skin allograft were first described in 1503 in the manuscripts of Branca of Sicily, and it has been practiced since World War II (8). The best source for skin allografts, specifically in burn treatments, is cadavers. Since living tissues have a risk of immunogenic rejection or virus transmission in the patient's body, it is used for short-term application to stimulate angiogenesis and to generate GFs and cytokines on scar tissue.

Xenografting, on the other hand, involves the transplantation of tissue from other animal species. It is used to supply collagen temporarily and promote regeneration in human scar tissue. Xenografting practices were first reported in Ebers papyrus in the 15th century BC, and the first application was carried out with frog skin (8). However, porcine skin is commonly preferred for burn treatment.

Amnion application has been used as a dressing for regional burns such as facial burns, since the 1910s. Amnions collected from the placentas of certain donors are preserved in tissue banks and used when demanded. Amnion is a structure rich in

collagen and some GFs, prevents the loss of protein, electrolytes and body fluids, soothes pain, reduces the risk of infection and accelerates the healing process of scar tissue. It differs from other techniques with the absence of any immunological markers and its antibacterial properties (8).

After decades of applying conventional wound healing and tissue regeneration methods, more technological and functional techniques such as cell coculture, cultured epithelial autografts, tissue engineered skin substitutes have been established within the scope of tissue engineering. In these techniques, differentiated, embryonic or induced pluripotent stem cells such as human dermal fibroblasts, hair follicle stem cells, bone marrow-derived mesenchymal stem cells, foreskin derived keratinocytes, keratinocyte stem cells, angiogenic endothelial progenitor cells, adipose tissue derived stem cells are used.

Tissue engineered skin substitutes stimulate the secretion of GFs and cytokines, thereby accelerating the healing of scar tissue and protecting the wound from external factors, while preventing tissue dehydration and the risk of infection. ‘Gauze’, which was first represented by Joseph Gamgee in 1880 as a traditional wound healing technique and applied as a skin substitute, is an absorbent dressing formed as a cotton wool sandwich (9). Similarly, Mangoldt described the technique of ‘epithelial cell transplantation’ in 1895 for the treatment of chronic wounds (10). This procedure involves obtaining epithelial cells from superficial epithelial tissue and seeding them into damaged skin tissue.

Tissue engineered skin substitutes are mainly composed of cells and ECM. Ideal skin substitutes should be sterile, serve as a barrier on the scar tissue, have a low level of inflammatory response, have no risk of toxicity, and allow water transmission through the native skin tissue. Besides, it should be easily produced, low-cost, resistant to stress, should be designed with minimal storage conditions and long-term shelf life,

and should be developed in a way that can adapt to irregular scar tissue and allow angiogenesis (8). If they are applied with biomaterials, they should be biodegradable, biocompatible, non-toxic, repairable, non-immunogenic, non-inflammatory, and the risk of disease transmission should be low.

Tissue engineered skin substitutes hold promise for future tissue regeneration and wound healing therapeutics. However, these substitutes require to be developed to promote vascularization in order to prolong their effective time and improve interactions with the host tissue. Furthermore, the standardization of preservation conditions is also highly important in terms of prolonging the effective time of the substitutes.

2.2.1. Types of bioengineered skin substitutes and features

Skin substitutes presently used are classified in various ways. They are classified as permanent, semi-permanent or temporary, according to the duration of covering the wound tissue. First of all, according to their composition, they are classified as cellular and acellular (11). According to their architectural structures, they are classified as epidermal, dermal or dermo-epidermal (composite) and depending on the types of biomaterials used cooperatively, they are classified as biological (autologous, allogenic, xenogeneic) or synthetic (biodegradable, non-biodegradable).

Acellular skin substitutes started to be applied as a temporary substitute towards the end of the 1970s for the treatment of superficial or mid-dermal partial thickness wounds and burns (10). Commonly, they consist of a nylon mesh or collagen that functions as the dermis and a silicone membrane that functions as the epidermis (8). Commercially available acellular skin substitutes are Alloderm® (LifeCell Inc.) which is used for repair of soft tissue defects, made from decellularized donor skin and only

matrix components remain to prevents immune response (12), OASIS Wound Matrix (Cook Biotech Inc.) (13), Biobrane® (Mylan Bertek Pharmeceuticals) which is used as a temporary dressing for burns awaiting placement of autografting (14), Integra® DRT (Integra® LifeSciences Corp.) is used for deep burn wounds and degrades when the host's cells invade and proliferate within it (31).

Autologous cells can be challenging to obtain from elderly or individuals with various diseases, therefore it is limiting. In such situations, the cells collected are expanded with cell culture techniques and proper treatments are developed for the patient. Optimizing the culture conditions is crucial to reduce cellular response and is provided by a wide variety of parameters such as oxygen support, GFs, ECM, and proximity to living tissue. Cultured autologous keratinocytes used in cellular autologous skin substitute applications provide a more permanent and functional skin covering. There are two types of autologous skin substitutes: cultured epidermal autograft (CEA) and cultured skin substitutes (CSS) (11). In CEA applications, autologous keratinocytes collected from the skin biopsy of the patient are cultured and grafted to the patient. In CSS applications, an autologous graft containing both epidermal and dermal components is produced. However, it is relatively more expensive and the preparation process is longer. In CSS applications, hyaluronic acid is often used, and such designs stimulate the expansion and migration of fibroblasts, control osmoregulation and matrix hydration, as well as support cleanse free radicals and regulate inflammation. However, a drawback of cultured skin substitutes is incomplete or irregular pigmentation. Under normal conditions, epidermal melanocytes, which both aim to protect the body from UV irradiations and affect the body visually for personal characteristics, establish a uniform distribution in the tissue and provide pigmentation. In some situations, cultured keratinocytes can permanently prevent pigmentation. These complications can be fixed by better understanding the structure and functions of melanocytes in prospective researches (36).

Cellular allogenic skin substitutes are mainly performed with living neonatal foreskin fibroblasts in the form of mesh or matrix. They are also used in the treatment of venous and diabetic ulcers, as well as wound healing in epidermolysis bullosa, skin cancer and various burns (11). In addition to these advances, autologous skin grafting cannot be used widely due to the difficulty of obtaining cells. Further, fibroblasts and keratinocytes, which are frequently used in skin substitutes, limit studies because these cell types cannot fully replace native tissue. Therefore, ongoing studies focus on different cell types. For instance, endothelial cells are studied for *in vitro* angiogenesis because vascular improvement develops more slowly in engineered tissues than in skin autografts (36).

Another cell source used in skin substitute studies is progenitor cells, which are formed as a result of the division of stem cells and enable them to differentiate into specific tissues. However, most differentiated progenitor cells have limited self-renewal capacities. Many researches on stem cell plasticity are carried out due to the limited proliferation of autologous stem cells in cellular skin substitutes applications. Within the scope of these researches, multi- and pluripotent stem cells are isolated from specific parts of the body. Stem cells have a high proliferation capacity and self-renewal ability, as well as a multi-lineage differentiation capacity, but they are not yet differentiated cells. During blastocyst formation, cells derived from the inner cell mass are pluripotent stem cells and are called embryonic stem cells (ESCs). ESCs can differentiate into any tissue from the three germ layers but are not capable of developing a new embryo. Other type is multipotent stem cells are obtained from mature tissues and are called adult stem cells (ASCs). Apart from these two fundamental stem cell groups, induced pluripotent stem cells (iPSC) developed by scientists are also used *in vitro* applications. iPSC is a new opportunity as it does not lead to ethical issues as a cell source in skin tissue engineering. However, it is not yet used in clinical applications due to the risk of triggering tumor growth (34).

The optimal source of progenitor cells utilized in cellular skin substitutes should be easily expandable, non-immunogenic, and capable of providing differentiation within the transplanted tissue. Cell resources significantly affect the application of engineered substitutes. Three main sources of cells are used: autologous (cells from the patient's body), allogenic (cells from another individual), and xenogeneic (cells from other animals). Xenogeneic cells are the least reliable because the risk of immune rejection and virus transmission is relatively high.

Some of commercially available epidermal skin substitutes are EpiDex (Modex Therapeutiques) which is consist of cultured keratinocytes from outer root sheath of scalp hair follicles to heal chronic leg ulcers (15), Epicel® (Genzyme Biosurgery) which is consist of sheets of autologous keratinocytes attached to petrolatum gauze support for full-thickness burns, however, since it is produced from cells harvested from the patient, its lack of off-the-shelf availability and its usage is limited since it does not contain dermis (16), Lyphoderm (XCELLentis) which is consist of lyophilized neonatal keratinocytes (17).

Some of commercially available dermal skin substitutes are denovoDerm™ (EUROSKINGRAFT) which is autologous dermal substitute (18), Dermagraft® (Advanced BioHealing, Inc.) as an allogenic type which is consist of bioabsorbable polyglactin mesh matrix seeded with human neonatal fibroblasts and cryopreserved and utilized to cover diabetic foot ulcers (29), TransCyte® (Advanced BioHealing, Inc.) as an allogenic type which is consist of collagen-coated nylon mesh seeded with neonatal human foreskin fibroblasts, however the product is stored frozen and thawed when used, this leads cells to die, the ECM and GFs remain effective (30), Matriderm (Dr Suwelack Skin and HealthCare AG) as a xenogeneic type which is consist of bovine non-crosslinked lyophilized dermis, coated with an elastin hydrolysate (19), Hybrid nanofibrous PLGA/chitosan membrane (Tianjin University) as a synthetic type which is PLGA/chitosan hybrid electrospun nanofibrous membrane (20).

Some of commercially available dermo-epidermal (composite) skin substitutes are TissueTech Autograft System (Laserskin and Hyalograft 3D) (Fidia Advanced Biopolymers) which is consist of cultured autologous keratinocytes and fibroblasts microperforated hyaluronic acid membrane (HAM) (19), OrCel® (Ortec International Inc.) as an allogenic type which is consist of type I collagen matrix seeded with neonatal foreskin fibroblasts and keratinocytes for treatment of donor sites with burns and surgical wounds (21), Oasis® (Healthpoint Biotherapeutics) as a xenogeneic type which is intact matrix from porcine small-intestine submucosa and intended for wound closure stimulation in acute, chronic and burns wounds (19).

Cellular skin substitutes, which are widely used in regenerative medicine, basically involve the production of structures called scaffolds produced with using various biomaterials. Scaffolds are primarily designed to provide a temporary matrix and their constructions are designed depending on the cell types to be used for particular treatments. These cell-laden scaffolds also play an important role as they enable 3D *in vitro* cell culture applications.

3D cell culture applications can more successfully reflect *in vivo* environmental conditions compared to monolayer cell culture, therefore the effects on the cellular activities and metabolic functions of the cells used with tissue engineered biomaterials can be examined in the most pragmatic way. The development of 3D cell culture technology will lead to more physiologically relevant procedures to better understanding of organogenesis and tissue morphology, the produce of new cellular therapies, the increase of cell-based testings, and the reduction of animal experiments. In order to investigate the serious side effects of drugs, researches on isolated cancer cells, specifically in chemotherapy drugs, can be detected and uncertain treatments can be prevented (26).

The focus of tissue engineering is to eliminate the limitations of conventional treatment approaches, studies on the production procedures of artificial tissues and organs that do not lead to immunological responses and provide a stable treatment. In skin tissue engineering applications, researches are carried out on components that can accelerate healing, relieve pain and regulate the conditions of the healing process. For these purposes, tissue engineering applications can basically be divided into four main groups:

- Tissue scaffolds
- GFs that promote healing
- Stem cells
- Gene therapy, which has been included in the scope of tissue engineering in recent years (32).

2.3. Scaffolds Used in Skin Tissue Engineering Applications

Scaffolds are extremely important application in tissue engineering that primarily provide structural support and used as a temporary artificial matrix to support cell growth and metabolism in transplanted tissue or *in vitro* studies (6). Scaffolds, which can also be utilized as carriers for drugs and GFs, have multi-tasking capability by serving as an ECM for cells at the same time. There are crucial features such as biocompatibility, biodegradability, degradation products should not induce an immune response, cytocompatibility, controllable degradation rate, showing the mechanical properties of the tissue it replaces, and having a porous structure for cellular functions that should be considered in the scaffold design (28). Well-designed scaffolds also support cell adhesion, stimulate cell growth, and allow differentiated cell functions, maintaining a balance between proper mechanical properties of porous structure and vascularization at the site of implantation (46).

Tissue engineered cell-laden scaffolds can be carried out in two ways: seeding cells into pre-manufactured porous scaffold structures or combining cells and polymers during scaffold production to provide cell encapsulation (33).

The ideal scaffold should be designed in a porous architecture to provide a sufficient environment for cell migration, growth and proliferation. Additionally, certainly important parameters such as pore size and distribution, surface properties which are crucial for cellular responses, interact with specific adhesion molecules, and stimulate their expansion and differentiation by regulating the movements of progenitor cells, directly influence 3D scaffold systems (5). The use of these 3D and porous scaffolds has significant advantages, such as being able to reflect the natural tissue and its micro environment at the macroscopic or cellular level (5).

Scaffolds made from biodegradable polymers hold great potential in the research of bioartificial tissues *in vitro*. Besides, selection of compatible components is highly essential in scaffold production by the reason of it plays a critical role in cellular growth. Thus, scaffolds can be produced with suitable substances in three ways as natural-derived, synthetic or hybrid, and these biomaterials used in their design are divided into three groups as polymers, ceramics and composite. Scaffolds produced from natural materials consist of proteins and carbohydrates obtained from plants or animals. However, scaffolds made from synthetic polymers are more robust than those natural-derived, and a wider variety of fabrication techniques can achieve physical properties on a larger scale. Besides, more reliable sources of synthetic raw materials are available (32).

The biggest limitation in scaffold structures is vascularization, which is related to the supply of oxygen and nutrients. It is almost inconceivable for vascularization to develop, specifically *in vitro* studies. However, *in vivo* vascularization may be achievable if proper stimulation and conditions are presented.

2.3.1. Types of scaffolds

Scaffolds, which play a crucial role in the treatment and, more importantly, regeneration of tissues, provide a suitable environment for cells to survive, proliferate and differentiate. Scaffolds developed by the combination of more than one polymer are very important because they have more functions than scaffolds obtained from a single polymer (3). They can be designed as degradable or non-degradable, from synthetic or naturally derived polymers.

Various techniques have been used to construct scaffold systems, such as using cell sheets formation by secreting ECM (Figure 1.1), pre-prepared porous scaffolds (Figure 1.2), decellularized ECM scaffolds (Figure 1.3), and cell encapsulation in hydrogel (Figure 1.4) (37). Apart from these main procedures, scaffolds are also achieved by phase separation, solid freeform production, electrospinning, bioprinting, porogen leaching, freeze drying, centrifugal casting, scaffold templating technique, micro-pattern application and gelation methods (46). In accordance with these techniques, they are basically divided into six groups:

- Porous scaffolds
- Fibrous scaffolds
- Acellular scaffolds
- Hydrogel scaffolds
- Microsphere scaffolds
- Composite scaffolds

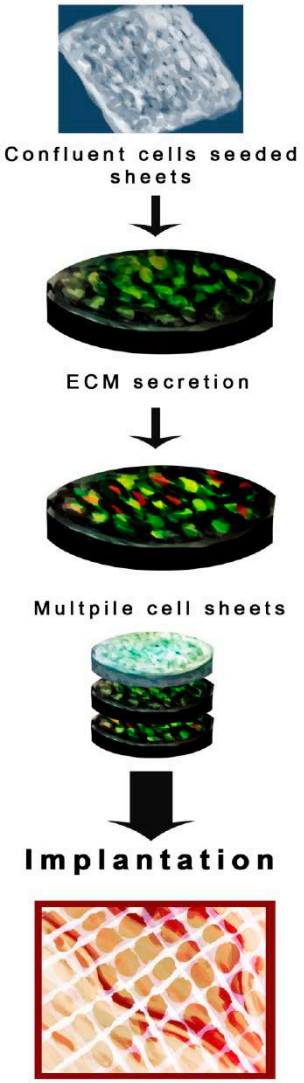

Figure 1.1. Cell sheets secreting ECM (37).

Cells seeded on cell sheet secrete ECM which will regulate expansion and proliferation.

Porous scaffolds produced from biodegradable, synthetic, natural or composite biomaterials, demonstrated in Figure 1.2, can be established in forms such as sponge, foam, and mesh. In order to achieve these porous formations, the use of porogens to adjust the desired pore size and shape, prototyping, layer-by-layer arrangement of nanofibers using electrospinning, freeze drying and 3D bioprinting techniques, which is the most up-to-date method, are used (37). The porous structure of the scaffold and

the design of these pore sizes in ideal sizes allow the cells to be seeded in the scaffold, supply them nutrients and oxygen, and manage their vital functions. Scaffolds produced in foam and sponge formation are more durable than mesh form. Such scaffolds are commonly produced by seeding keratinocytes or fibroblasts into collagen-based architectures. OrCel®, one of the commercially available products, which was given an example before in this thesis (2.2.1.), was obtained as a porous sponge structure by utilizing bilayered type-I bovine collagen.

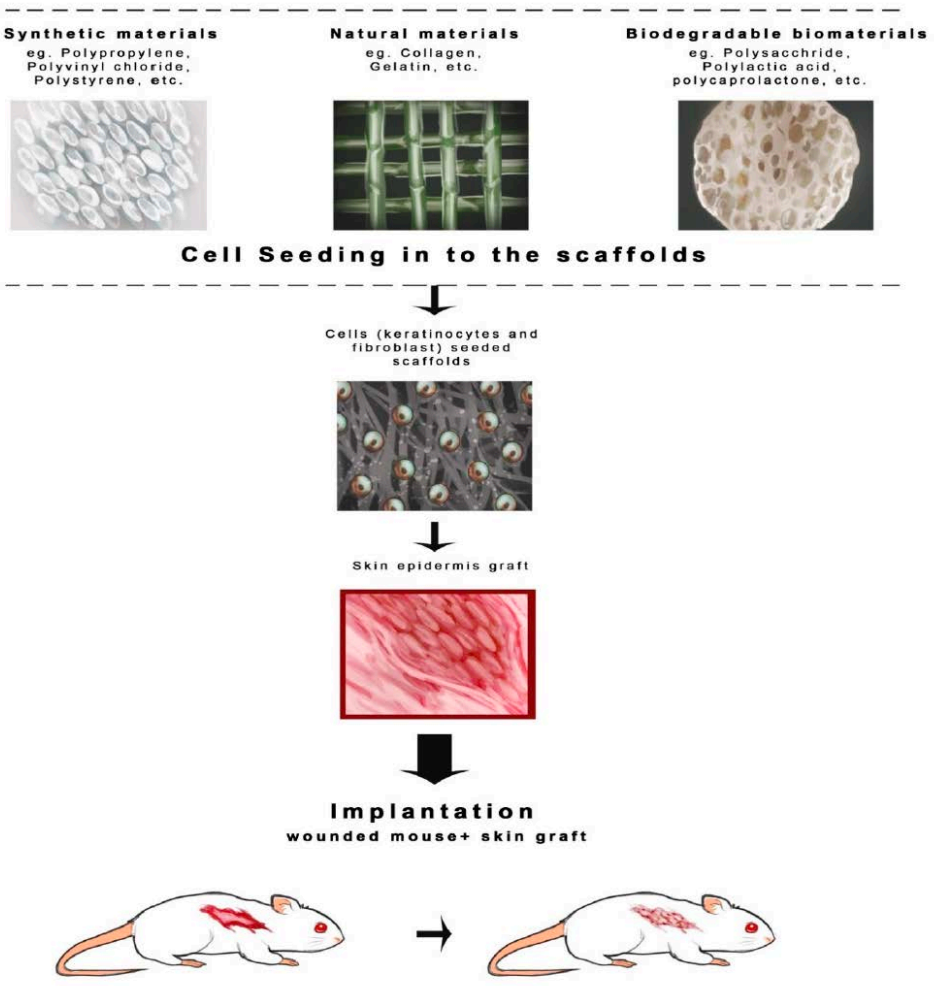

Figure 1.2. Cell seeded porous scaffolds (37).

Porous scaffold designs that support cellular functions are achieved by utilizing numerous natural, synthetic and biodegradable substances. These systems support to maintain nutritional components to skin cells such as keratinocytes and fibroblasts, thus these scaffolds can be used for wound replacement.

Fibrous scaffolds are made of both natural-derived and synthetic polymers and actually belong to the porous scaffold class and are constructed from nanofibers by techniques such as electrospinning, drawing, self-assembly, template synthesis, and phase separation. They are highly successful systems in expressing the natural tissue properties and providing an environment for cellular activities. It can be used in both hard and soft tissue applications and as a controllable drug delivery tool. Porous fibrous scaffolds are particularly effective in resemblance of fibril structures to the ECM, management of nutrient molecules and metabolic wastes, and prevention of microorganisms from sheltering in small pores. In fact, it has been observed that cellular functions are performed better in fibrous structures obtained from chitosan than sponges designed by 3D bioprinting (37). Biobrane® and Integra® are in this group among commercially available products, which have been exemplified earlier in this thesis (2.2.1.).

Acellular scaffolds are produced from the collagen matrices collected after the cellular components are removed from the tissues. When these scaffolds are transplanted into wound tissue, as shown in Figure 1.3, they gradually degrade and are replaced by secreted ECM proteins. Fibrous scaffolds derived from tissues that have been removed with this respect, numerous cells can be expanded on the scaffold for specific purposes. These decellularization procedures rely on chemical, physical and enzymatic degradations such as repeated freeze-thaw periods, trypsin/EDTA treatment, hypertonic or hypotonic solution treatment. With these approaches, it is desired that the biochemical and physical properties of the decellularized structure resemble natural tissue, therefore, it can be adequately grown in the damaged tissue. Compared to alternative scaffold formations, the advantage of acellular scaffolds is that it is easier for cells to attach to the ECM structure. Thus, tissue improvement is achieved similar to natural tissue and the risk of immunological response is low in terms of long-term treatment. In the field of skin regeneration, purification of cells and antigen components and using dermal allografts are commonly used for organs such as the heart and urinary bladder (37). Alloderm® is one of the commercially available acellular allograft products, as exemplified earlier in this thesis (2.2.1.).

Hydrogel scaffolds produced from natural or synthetic polymers have an extremely important potential in tissue engineering due to their great biocompatibility, biodegradability and ability to interact with cells. Hydrogel designs are advanced significantly in controllable drug delivery studies (37). Hydrogels, which are developed by mixing cells with a monomeric solution of a synthetic polymer shown in Figure 1.4, are usually achieved by crosslinking ionic or covalently. The most remarkable feature in hydrogel formation is that biomaterials can self-assembly from liquid monomeric phase to solid polymeric network construction under the influence of various factors, such as pH and temperature. An ideal hydrogel scaffold should have great degradation, reproducible and tunable characteristics such as angiogenesis, wound healing, and cell differentiation. In the construction of hydrogel scaffolds developed for skin regeneration, mostly hyaluronic acid-fibronectin and chitosan-gelatin combinations with PLGA nanofibrous structure are selected. Furthermore, dextran-based hydrogels are also highly efficient in skin regeneration and tissue healing. Hydrogels achieved by seeding keratinocytes on photo-crosslinkable gelatin hydrogels are also applied as epidermal substitute and dressing (41).

Microsphere scaffold structures, which have been established in recent years, are mainly utilized in gene therapy and drug delivery applications because they provide longer and slower release of encapsulated drugs (37). Microsphere scaffolds are commonly obtained from a mixture of various polymers and drug delivery processes are adjusted based on the molecular weight (MW) of the polymers. They can be produced easily by procedures such as heat sintering, solvent vapor treatment, solvent/non-solvent or non-solvent-only sintering (37). In particular, nanoparticle-based microspheres-derived from PLGA and natural polymers such as collagen or gelatin are being developed as dermal scaffolds for drug delivery applications such as antibiotics or GFs. In a recent study, PLGA-based microspheres were performed with gentamicin, generating an antibacterial effect against Staphylococcus bacteria, effectively providing adhesion and proliferation of fibroblast cells (38). At the same time, microsphere scaffolds seeded with MSCs (mesenchymal stem cells) are utilized for skin regeneration and have been shown to have an effect on cutaneous wound

healing and reconstruction of sweat glands (39). Gelatin-based microsphere scaffolds are also used as carriers for stem cells in skin regeneration (40). In addition to these applications, studies with other polymers are still ongoing.

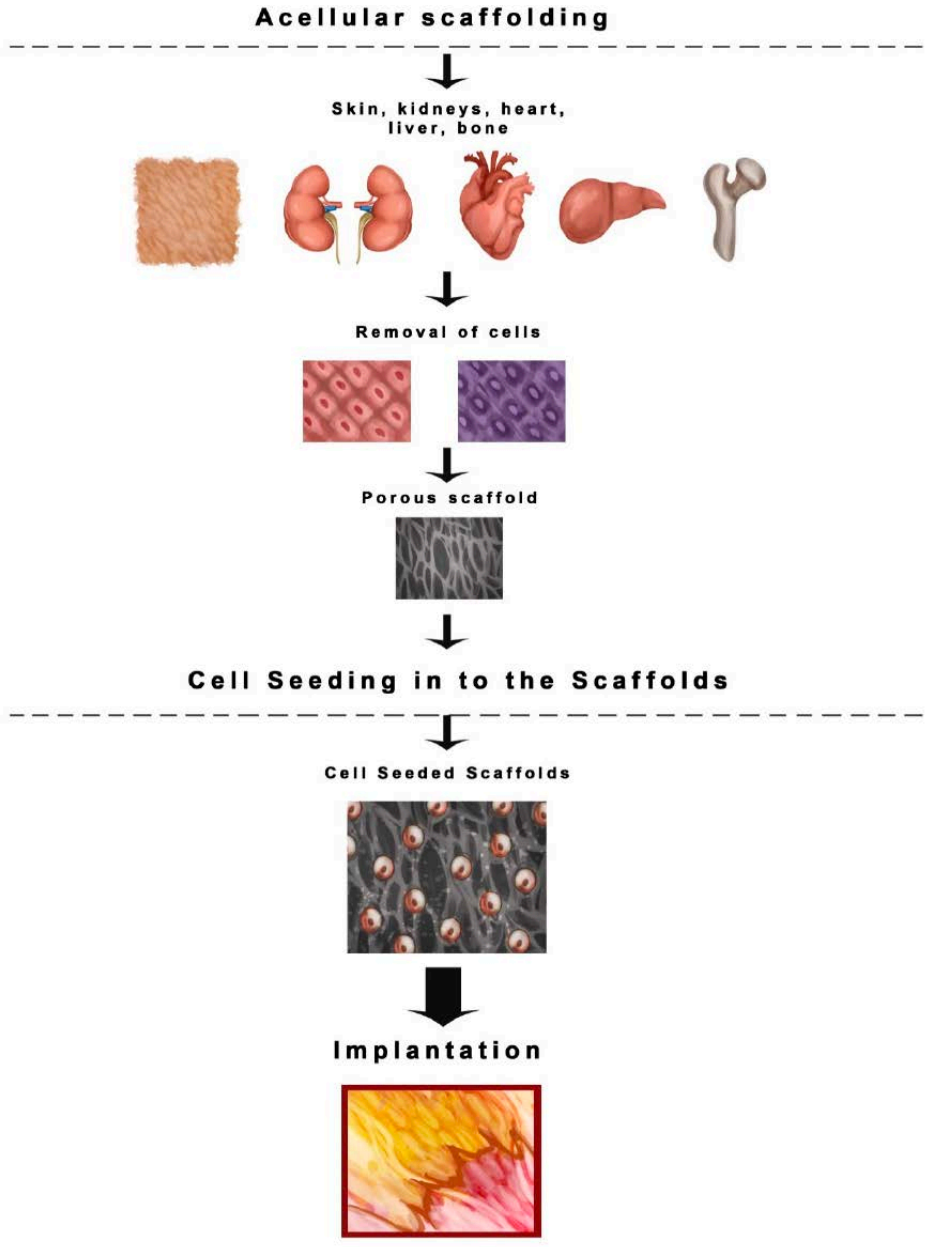

Figure 1.3. Decellularized ECM scaffolds (37).

In this application, the cells of the organ are completely removed to achieve ECM-based matrices. Many cell types can grow effectively in such scaffold structures.

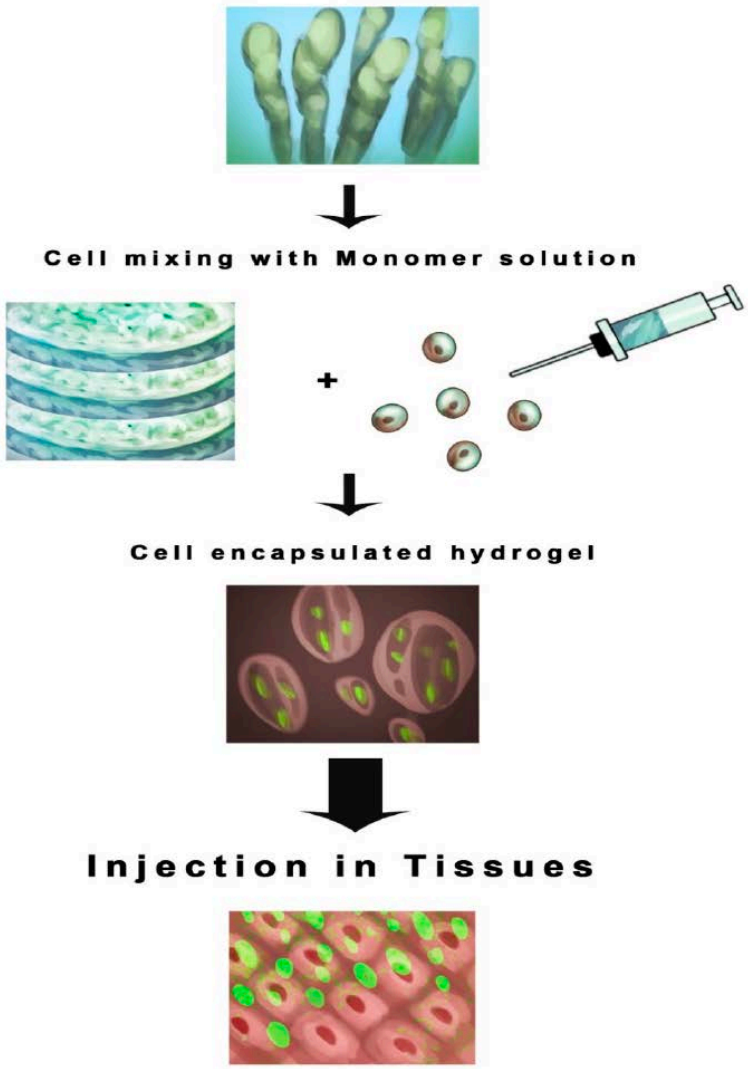

Figure 1.4. Hydrogels with cells (37).

Skin cells such as keratinocytes and fibroblasts mixed with a monomeric polymer solution are applied to the wound tissue of injectable hydrogels to stimulate healing and regeneration.

The material properties of polymer-bioceramic composite scaffold structures can manage the physiological responses of the host tissue. The bioceramic description comes from its use, specifically in bone tissue regeneration. There are mainly three scaffold production techniques using ceramics: non-absorbable and inert such as zirconia, alumina, silicone, semi-inert such as glass ceramic and hydroxyapatite (HAP) and non-inert biodegradable such as aluminum calcium phosphate, tricalcium

phosphate, etc. The use of ceramics in the scaffold design has advantages such as compatibility, resistance to corrosion and high pressure. In addition to these, it has important drawbacks such as being difficult to shape, being brittle, not reliable and having a dense structure (37). On the other hand, polymers have a flexible structure on their own, but they are not mechanically durable and stiff, besides ceramics are stiff. Thus, the mechanical properties of scaffolds obtained from polymer-bioceramics as composite are more advanced, stiffer and more durable. This composite structure reduces the degradation rate of the polymer. The porous scaffold structure consisting of HAP/PLGA shows excellent mechanical properties and biocompatibility (42). There are considerations on the use of bioactive glasses in soft tissue engineering due to their angiogenic potential (43). As a result of these researches, 45S5 Bioglass® was produced as a PLGA-mesh bioactive glass production, which demonstrated significant improvements in performing neovascularization of scaffolds both *in vitro* and *in vivo* (43). Bioactive glass component is biocompatible in terms of maintaining a suitable environment for tissue growth. It was observed that poly(ethylene oxide) mesoporous bioactive glass (MBG) fibers produced by the electro-spun technique support regenerated tissues and release anti-inflammatory drugs (44). In addition, composite films made of MBG-chitosan also have potential for skin regeneration.

2.4. Hydrogel Scaffolds Used in Skin Tissue Engineering

Hydrogel scaffold is the first biomaterial designed for use in the human body which was first introduced in 1954 by Wichterle and Lim when the first synthetic hydrogels were performed (45). Hydrogels are 3D tissue-like hydrophilic polymeric network organizations that are insoluble, crosslinkable and be able to swallow extremely high amounts of water and biological fluids with natural or synthetic polymer networks. They can have water composition of over 90% and can be 10-1000% times its dry weight (48). Hydrogel scaffolds with high biocompatibility, mimic the natural structure of ECM, present excellent mechanical properties similar to soft tissues, support cellular activities and can reflect the *in vivo* environment very

close to natural tissue. The mechanical properties of hydrogels have a highly important role in their mechanical stability against external forces as well as in cellular interactions, including differentiation of stem cells (3). Altering the composition of the hydrogel influences swelling behaviors and rheological properties (58). Owing to these properties, it is one of the most promising materials in cell culture applications and regenerative medicine (48).

Hydrogels are designed to be utilized in the form of bandages, adhesives and burn dressings for the healing of skin injuries. Hydrogel dressing protects the wound from microbial contamination, prevents the loss of body fluids, provides oxygen flow to the wound and usually accelerate the healing process. Wound dressing hydrogels performed with natural materials are seen as a potential choice in wound covering due to their non-toxicity, biocompatible, biodegradable and likewise their insolubility, hydrophilicity and excellent swelling behavior (58).

Hydrogels are divided into three groups according to their physical properties as solid, semi-solid and liquid (46). Solid hydrogels, which are solid at RT but can be dissolved in water, buffer solutions and biological fluids, can provide a sufficient cellular environment by mimicking the physical, chemical, electrical and biological properties of the most complex tissues. In the production of some hydrogels, gel form may not be achieved by directly mixing two or more components (26). Together with ionic or covalent crosslinkers, it can form stable crosslinked networks. Semi-solid hydrogels, also called bio-adhesive or muco-adhesive, are carried out highly durable by interfacial forces such as hydrogen bonds, van der Waals interactions and electrostatics, thereby improving their adhesive properties. It is utilized in applications such as drug delivery and efficient dosage treatments in medical fields such as buccal, rectal, vaginal, ocular, sublingual and nasal routes. It is developed from two distinct polymers, one of which is a high-molecular-weight polymer that necessarily presents biological activities. It can be applied by injection method due to its hydrophilic properties. Liquid hydrogels, which are liquid at RT but can turn into elastic, like soft

tissue with good functions when appropriate temperatures are applied, are quite flexible structures and can adapt their systems with environmental factors. It can be applied effortlessly with inorganic and organic components, drugs, proteins and cells without the demand for surgery.

Apart from these three groups, there are also superabsorbent hybrid hydrogels (SHH) with extraordinary water absorption capacity obtained from superabsorbent polymers (46). Common polymers absorb up to 100% (1g/g), while SHH scaffolds can absorb up to 1k-100k% (10-1000g/g) of deionized water. Yang et al. has declared that it can be a very sufficient system for agricultural applications. It can be applied as a water conservator in dry or desert environments. It can also help remove pollutants from the environment by absorbing toxic heavy ions and dyes. It is widely utilized in products that entrap liquid, such as baby diapers and feminine sanitary napkins.

2.4.1. Properties of hydrogel scaffolds

To better understand the role of cellular microenvironments *in vitro*, synthetic ECMs that can reflect native ECM microstructures and properties have been developed utilizing various biomaterials. Hydrogel scaffolds are produced by chemical or physical crosslinker have been a significant tool for cell culture studies. Hydrogel scaffolds contain high water content like natural ECM. Gelation kinetics and hydrogel properties can be managed by various crosslinking reactions. Furthermore, it is an exceptional application in allowing 3D cell culture to demonstrate *in vivo* cellular environment properties (56).

Mechanical properties such as stiffness and swelling can be managed by chemical modifications of polymers, as physical properties such as crosslinking type and density, and MW distribution. Gelation conditions and crosslinker type can also

influence mechanical properties. Temperature affects the gelling time. For instance, diffusion of Ca^{+2} ions may decrease at low temperature and crosslinking slows down. Increasing the polymer concentration enhances the stiffness, exemplarily in high-MW alginates, thus increasing the viscosity of the pre-gelled solution. However, this is an undesirable feature as it complicates the gelation process and the cells incorporated with polymers by mixing or injection are at risk of being damaged by high shear forces. This situation can be overcome by manipulation and distribution of the MW of the complemented polymers (3).

The degradation rate of hydrogels can be developed using enzymes, chemicals or water-sensitive functional groups. If the stiffness, which is inversely proportional to the pore size, decreases, the degradation rate increases. Therefore, if the pore size of the biomaterial is small in 3D cell culture, cells cannot proliferate. As the polymer concentration increases, stiffness increases, resulting in enhanced mechanical properties (56).

In order to obtain cell-laden hydrogels, the cells are first suspended in hydrogel precursors and entrapped by achieving the gel initiation reaction via crosslinkers (26). The crosslinker density used in cell-laden hydrogels can influence the cytotoxicity behavior. Similarly, the expansion of cells depends on the functional groups of the material and the degree of stiffness of the substrate. Increasing the stiffness reduces the pore size, which limits cell proliferation and migration. Consequently, the stiffness increases, proliferation significantly reduces (56).

2.5. Polymers Used in Hydrogel Design

Biomaterials and bioactive molecules utilized in tissue replacement and drug release applications, must be controllable, degradable and their mechanical properties

must be specifically designed. The degradation, which is obtained according to the MW and concentration of the polymer used in the production of biomaterials, provides space for the growing new tissue, supports the interaction of cells in the surrounding tissues and the release of incorporated bioactive molecules (3).

Biodegradable hydrogels are achieved by ionic (physical) crosslink techniques, and for their degradability these techniques are reversible reactions. During degradation, the hydrolytically or enzymatically susceptible linkages are broken, and the hydrogel continues to lose mass until all the crosslink bonds that provide the 3D scaffold structure are broken. If the degradation rate is too low, the hydrogel may disperse easily, and if the degradation rate is high, ECM accumulation will occur, affecting cellular functions. For these reasons, the selection of biomaterials to be used is critical. First of all, certain, reliable and bioinert materials are preferred, so that artificial scaffolds placed on the patient's body do not lead to cellular response (7).

2.5.1. Synthetic polymers

Synthetic polymers have advantages such as large water retention capacity, good permeability, high gel strength. Hydrogels designed to be stimuli-susceptible such as solvent, heat, UV irradiation, light, pressure, magnetism and pH in drug release systems are called smart gels (46). Hydrogels obtained from synthetic polymers are mechanically more robust than naturally derived ones but are not biocompatible *in vivo* and have a slower degradation rate (4).

Synthetic polymers frequently utilized in hydrogel composition are mainly poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), poly(lactic-co-glycolic acid) (PLGA or PLG), poly(hydroxyethyl methacrylate) (polyHEMA), poly(acrylic acid) (PAA) and its derivatives, poly(acrylate), poly(lactic acid) (PLA), poly(glycolic acid)

(PGA), poly(caprolactone) (PCL) and their copolymers, polyurethane, poly(acrylamide) (PA), poly(methacrylamide) and their derivatives, non-degradable poly(methyl methacrylate) (PMMA) etc. (60).

PVA is widely utilized in many medical applications such as drug delivery, artificial tears, and contact lenses as a water-soluble and perfectly biocompatible polymer that can form of a hydrogel with many techniques, especially covalent crosslinking (47). Fan et al. produced chitosan-gelatin-PVA based hydrogels as wound dressing, and found that the hemostatic properties of the hydrogels were at optimum level in the design with a chitosan:gelatin weight ratio of 1:1 (71). Scaffolds produced with polyHEMA are commonly used in dressing applications because they are effective in wound healing (46). PEG-based hydrogels are highly biocompatible and inert, characterized by their water-soluble properties, and are applied in drug releasing (59). On the other hand, polyurethanes are widely used as catheter coating material and as a pharmaceutical agent. Polymers such as PLGA and PCL have the advantage of minimal toxicity. However, in the practice of PLGA, there is a risk of leading to inflammation while the oligomers form (48).

Biomaterials obtained from synthetic polymers have deficiencies in forming a suitable architecture for cell attachment and hydrophobicity (6). They are degraded by hydrolysis and degradation products such as glycolic acid and lactic acid are harmful to cells and lead to inflammation.

2.5.2. Natural-derived polymers

Biopolymers are polymers that are biocompatible and obtained from natural sources such as microorganisms and plants and used in a wide variety of pharmaceutical and medical applications, including tissue engineering and

regenerative medicine (3). Biocompatibility and biodegradability properties are highly important in drug release systems at a particular time/region (46).

Natural polymers, which are bioactive, are very beneficial in cell culture studies as they involve cellular binding domains for cell adhesion and advanced phenotypic properties that can be managed with soluble signal factors (56). Also, their degradation is occurred by enzymes *in vivo* (48).

Biodegradable natural polymers are divided into two primary groups as peptide/protein and polysaccharide-based, there are also natural polymers classified as polyhydroxyalkanoates and polynucleotides (7).

2.5.2.1. Peptide/protein-based polymers

Peptides are short chains composed of dozens of amino acids while proteins are longer chains composed of hundreds of amino acids. Amino acids are hydrolytically linked by amide bonds therefore they can be degraded enzymatically. Their main drawback is that they cannot be formed by conventional methods as leading substance. They can also generate inherent immunogenicity because the peptide and proteins are at risk of being perceived as foreign by the patient's immune system. On the other hand, since it has remarkable biological properties, a wide variety of polymers with desired properties can be produced. Proteins frequently utilized in medical applications are collagen, gelatin, elastin, keratin, silk and proteoglycan.

Collagen is commonly obtained from cattle, fish and many other species and is a key component of tissue architecture. It provides mechanical stability, supports attachment and growth of cells, and also presents a biocompatible matrix for cell

transplantation. It is widely used as a tissue expander and bulking agent. Gelatin is obtained from partially hydrolyzed collagen and is broadly applied in the food industry. It is a scaffold component that has been extensively studied as a matrix in 3D cell culture. Elastin is isolated from elastic tissues of cattle and birds and provides flexibility to tissues as one of the key components of tissue formation. Silk is isolated from insect larvae and is broadly used in the textile industry due to its incredibly strong properties. It is also utilized as a scaffold component and cell culture substrate in tissue engineering. Proteoglycan is a major component of ECM and collected from a wide variety of tissue extracts. It is used to search cell-matrix and matrix-matrix interactions, cell proliferation and cell migration due to its role in highly important cellular pathways. They can be stabilized very easily applying heat, and can be performed with various pore sizes and degradation rates. An example of a commercially available product is the Integra, which has been referred earlier in this thesis (2.2.1.).

2.5.2.2. Polysaccharide-based polymers

Polysaccharide polymers, which are commonly utilized in nanoparticle and hydrogel stabilization, have properties such as non-toxicity, biocompatible, biodegradable, bio-stable, high water absorption capacity, forming a large variety of architectures with basic chemical modifications, abundant, susceptible to enzymatic digestions. Polymers such as alginate, hyaluronan and dextran, which are not animal-derived, carrageenan, chitosan, gellan gum, guar gum, pectin, cellulose, agarose and xanthan gum are in this group. Since other animal-derived biopolymers have the risk of pathogen transmission and immunogenicity, their usage in clinical applications is limited (26). Polysaccharide hydrogels can be efficiently produced both by physical techniques such as freeze-thaw and chemical techniques such as crosslinking. However, hydrogels produced by freeze-thaw technique indicate more advanced properties than hydrogels produced by chemical crosslinking. Due to its rheological properties, it is preferred especially in agro-food, paper, textile, cosmetics, biomedical

and pharmaceutical fields. Because it shows good compatibility, it is applied in skin, cartilage, bone and liver regeneration and applications to accelerate wound healing processes (46).

Cellulose is the basic structural component of plants and provides the stems, stalks and trunks rigidity. It is obtained from the cell walls of green plants. Starch can be obtained from all staple foods and is the most important energy store of plants. Alginate is derived from bacterial cell walls and protects bacteria against phagocytosis of white blood cells and harmful protozoa. To provide cell attachment, alginate must be modified using adhesive ligands. Ionic crosslinking with divalent cations provides cell encapsulation (55). Glycosaminoglycans are very common and play a crucial role in cell-matrix, matrix-matrix interactions and cell proliferation and migration. Hyaluronic acid, the largest glycosaminoglycan, can be obtained from natural sources as well as by microbial fermentation. Due to its water-binding capacity, dilute hyaluronic acid solutions form viscous solutions. It is non-antigenic, does not generate inflammatory or foreign body reaction, and is easy to isolate and modify, making it an effective glycosaminoglycan as a biomaterial. The main disadvantages are limited residence time and mechanical properties (7). Chitin/chitosan is a structural component and is derived from the exoskeleton of insects, shell of crustaceans and cell wall of fungi.

2.5.3. Alginic acid (alginate)

Alginic acid, also known as alginate (ALG), collected from brown seaweed, is utilized in the food industry as a mediator and emulsifier due to its interactions with oil and fibers, and in textiles and printing applications with the help of its high color yield. It is widely used in the pharmaceutical industry as an excipient in many applications, such as wound dressing, particularly due to its ability to provide moisture to the wound. It is a hydrophilic, biocompatible and non-immunogenic biopolymer

that can be used in various forms such as foam, hydrogel, nanofiber, film, membrane, sponge (26). The aqueous solutions of alginate, which is a soft and flexible polymer, have non-Newtonian properties. For instance, the viscosity decreases as the shear rate increases (3). In spite of their biological activities are not good, they are applied collectively with other components in tissue engineering applications and to develop hydrogel matrix to support cell adhesion and expansion. However, limited inherent cell adhesion and cellular interactions may be advantageous for cell encapsulation. Its degradation is not certain and its mechanical stability is dependent on the types of calcium chelates in it. When alginate is used with gelatin, whose properties can be improved depending on temperature, degradation becomes manageable (4). Furthermore, when alginate is applied together with gelatin, it can turn into an accomplished substrate in 3D cell culture due to biological properties such as biocompatibility, biodegradability and non-toxicity (26). It can be modified with adhesion molecules such as fibronectin, laminin and collagen to enhance cell adhesion (3). It also shows an ECM structure feature that retains GFs and other molecules *in vitro* applications. Alginate polymers are commonly used in beads, microporous scaffolds, and 3D printed scaffold structures. However, difficulties of alginate polymer used in tissue engineering applications such as not being enzymatically degraded and the rate of degradation cannot be managed.

The alginate molecule consists of repeating units of β-1,4-linked D-mannuronic acid (M) and L-guluronic acid (G) blocks. The distribution of these blocks is effective in the formation of ionic gelation, which enables it to develop different polymer chains by building bonds between divalent cations and G blocks. Viscosity increases when the presence of M blocks is high, which is an undesirable aspect in the gelling process. When the presence of G blocks is high, gelling properties advance and a stiffer structure is developed. Alginate is soluble in aqueous solutions and can be formed a stable construct by providing ionic interactions with divalent cations at RT. It can easily become insoluble by crosslinking and form a gel structure (8). Their high affinities for divalent cations are Ca^{2+}>Sr^{2+}>Ba^{2+}, respectively, and these ions influence elasticity, stability and swelling properties. While Ba^{2+} provides the best

advancement of mechanical properties, the use of Ca^{2+} has a significant effect on cell viability (4). Alginate can form stable, inherent, biodegradable and biocompatible hydrogel design together with Ca^{2+} ions with lower concentration than itself (46). It also has a capability of absorbing a lot of water due to the presence of carboxylic groups.

Gel elasticity depends on the gelation technique, either ionic or covalently. Since ionic crosslinking is reversible, the hydrogels achieved in this way show pH-dependent swelling properties. Furthermore, ionically crosslinked hydrogels become less stiff but more durable, while chemically crosslinked hydrogels become stiffer and brittle (8). Chemical crosslinkers are not preferred because they require the use of toxic components. Two different techniques, diffusion gelation and internal gelation, have been established to achieve gel form from alginate polymer. Diffusion gelation is commonly used in the preparation of beads and develops when ions diffuse from another source into the alginate solution. It is commonly applied by dripping $CaCl_2$ into alginate. In internal gelation, ions such as dissolved or suspended carbonate salts, calcium EDTA, calcium citrate, calcium sulfate are released into alginate in a controlled manner from an inert source.

Besides *in vitro* applications, chemically modified alginate polymers are widely utilized in wound healing, as carriers for transplanted cells, and in drug delivery to promote protein regeneration. On the grounds that they are a low-cost biomaterial, their mechanical properties can be easily regulated by physicochemical modifications in bioprinting, and they present excellent biocompatibility in cell immobilization (3). As an example of the use of polymers together as bioink in bioprinting, when the less rigid alginate and gelatin polymers develop a composite, a more rigid substance can be formed with the help of gelatin properties. They are also commonly used in multilayer bioprinting design, since alginate can crosslink rapidly and gelatin can develop temperature-dependent properties (4).

The most remarkable property of alginate hydrogels is that they do not adhere to the wound surface and do not lead to secondary damage when removed from the surface (8). Examples of products commercially available for alginate dressings are Algisite™ (Smith & Nephew) which is soothing wound contours and moisturizing wound tissue (61), Tegagen™ (Allegro Medicals) which is applied for diabetic and infected wounds (62), and Kaltostat® (ConvaTec) which is specified as an immediate dressing for chronic and acute wounds, and for wounds with slight bleeding (63).

Zhou et al. reported that the use of alginate polymer affects the water absorption capacity in the hydrogel they obtained using alginate, hyaluronic acid and gelatin polymers at various concentrations and ratios with EDC (1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide) as a crosslinker (25). Satish et al. reported that hydrogel scaffolds containing alginate, gelatin and PVA could clear exudates and provide a suitable environment to enhance wound healing (64). Zhang et al. produced a microcapsule hydrogel based on alginate and cellulose whiskers, which they obtained by crosslinking with Ca^{+2} ions. As a result, it has been declared that mechanical properties improve and drug release becomes stable without causing cytotoxicity (4). Datta et al. have shown that alginate can be efficient in reconstructive surgery and bone regeneration. Accordingly, alginate scaffolds support the formation of bone tissue (4). Furthermore, another study has shown that it can be applied as a porous hydrogel scaffold for cardiac muscle regeneration (65).

2.5.4. Hyaluronan (hyaluronic acid)

Hyaluronan, also known as hyaluronic acid, is secreted from the inner plasma membrane of cells by hyaluronan synthases, a transmembrane protein, and presented to the ECM. It is the primary component of the ECM and plays a significant role in the development of many tissues, such as skin, cartilage and brain, and wound healing. Due to its ability to grow up to 1000 times its weight and its highly flexible structure,

it provides resistance to tissues against trauma and deformations. Hyaluronic acid, a naturally derived, non-sulfated glycosaminoglycan, composed of a repeating unit of N-acetyl D-glucosamine and D-glucuronic acid (50). It can be obtained from various tissues of many animals and from *Escherichia coli* by microbial fermentation. It is a negatively charged molecule and interacts with the hydrogen bonds of water. As with the alginate molecular structure, it can crosslink due to the presence of carboxylic acid and allows chemical modifications by providing the formation of cellular attachment domains. It is also a hydrophilic molecule that can form gel-like structures in aqueous solutions due to the presence of hydroxyl groups (54). Since the physiological reactions of the molecules depend on the hyaluronic acid concentration and molecular weight, their cellular activities play a key role in angiogenesis, wound healing and tissue regeneration. Due to their excellent hydrophilic, viscoelastic, and mucoadhesive properties, they are used to support cellular functions in a wide variety of medical applications such as drug delivery, controlled release of drugs, GFs, and antibodies, *in vitro* fertilization to protect embryo, eye surgery, facial or intradermal implants, and tissue regeneration (23).

It is a widely used in tissue engineering biomaterial due to its non-adhesive, non-immunogenic, non-allergic, non-thrombogenic, biocompatible, bioactive, manageable viscoelasticity and adjustable mechanical properties (53). However, it has a drawback that it degrades easily. It can be utilized *in vitro* and medical applications by crosslinking with various biomolecules such as aldehyde, thiols, methacrylates, and dihydrazide groups in order to decrease the degradation rate and enhance its stability by improving its rheological properties (50).

Hyaluronic acid polymers are used to design scaffolds with gelation, electrospinning and freeze drying techniques. Freeze drying is applied to hydrogel synthesized with gelation, and porous structures can be achieved by sublimation of dense ice masses (24). Accordingly, high usage of hyaluronic acid in composite

hydrogels provides structures with larger pores, thus promoting cell migration, tissue growth and diffusion of molecules (54).

Hyaluronic acid, which is highly efficient in the development of cellular interactions, has very important advantages with its biological relevance, mechanical integrity, and chemically adjustable properties, particularly in designing hydrogels (5). By increasing the matrix stiffness in hydrogel scaffolds, it provides the improvement of flexibility and hydrodynamic properties. Furthermore, it is applied with cells and GFs in the scaffolds produced in order to eliminate the issues related with autologous and allogenic grafting (54).

The practice of hyaluronic acid to improve vocal fold regeneration has been shown to support regulation of the ECM and promote wound healing, due to the resemblance of its rheological properties with the lamina propria (24). Additionally, its usage with alginate polymer makes hyaluronic acid more stable in PBS and likewise supports stem cell viability and differentiation *in vivo*.

Thompson et al. have shown that when hydrogels achieved by adding copolymer to pure hyaluronic acid are analyzed, it supports cell penetration and tissues more. It was also noticed that the mechanical properties enhanced with the increased crosslink (54).

2.5.5. Gelatin

Gelatin is obtained by hydrolysis of the natural triple helix construction as a result of the denaturation of collagen and the formation of single-chain molecules (50). It is a highly delicate polymer collected mostly from connective and bone tissues such as

porcine, bovine and fish collagen. Gelatin is commonly utilized in biomedical applications to support cell migration, cell adhesion, cell proliferation and differentiation due to its non-immunogenic, biocompatible, biodegradable, non-toxicity properties (4). In addition to tissue engineering applications such as wound healing, drug delivery, pharmacy, gene therapy, regenerative medicine, it is also a frequently preferred polymer in the food industry (4).

Gelatin has drawbacks such as low stiff structure, being a soluble biomaterial since it can change its state from gel to solution at 30-40^{o} C, and consequently limited long-term stability (48). Since it turns into solution reversibly with temperature, it is needed to perform crosslinking with chemical modifications (4). The gel formation of gelatin is usually achieved by aldehyde crosslinking (51). However, it is challenging to obtain a stiffer material since chemical crosslinking applications affect the degradation rate of gelatin. In addition to these methods, methacrylate is applied to promote crosslinking by exposure to UV irradiation, and the obtained methacrylate gelatin (GEL-MA) can interact with various cell lines as a bioactive component (52).

Depending on the technique of obtaining from collagen, the electrical charge of gelatin polymer, which can be acidic or alkaline, regulates the binding of therapeutic agents, permitting it to be used effectively in controlled drug release applications and in the transport of GFs in bone regeneration (48). Since it has positively charged residues, such as arginine and lysine, it facilitates the GFs and ECM components to function collectively (25). Yamada et al. successfully implanted a hydrogel, in which alkaline fibroblast GFs were placed in acidic gelatin, on the injured rabbit skull (66).

Gelatin, which is the most practiced polymer in cell culture, provides a highly suitable environment to support cell adhesion, migration, differentiation and proliferation (4). It can mimic the structure of the ECM, therefore it can be utilized as a tissue model for testing developed pharmaceuticals recently. Furthermore, gelatin,

which is a very useful substance for bioprinting and designing organ models, is also applied for coating application (52).

Chung et al., performed a sodium alginate-gelatin-based hydrogel and examined its effects on cell growth. As a result of this study, it was observed that the gelatin polymer strengthened the cell-seeded alginate scaffolds (67).

Matrigel® (Corning Life Sciences), one of the commercially available alginate-gelatin-based hydrogel products, is used as a base matrix for stem cells, allowing stem cells to remain undifferentiated (4). Another commercially available product, PuraMatrix™ (Corning™) is a peptide hydrogel containing amino acids sufficient for 3D cell culture (68).

Crosslinking of hydrogels

Hydrogel scaffolds need to be crosslinked in order not to degrade quickly at body temperature. The polymers form 3D networks by connecting to each other with the application of crosslinking, and the polymers involved lose the properties of their single-chain states to stabilize the formed hydrogel. Crosslinked polymers become more mechanically stable but less flexible and degrade more rapidly depending on temperature and solvent (4).

It is necessary to keep the polymerization time short and to use nontoxic initiators for the crosslinking of hydrogels to be applied with cells. Since the mechanical properties of hydrogels can influence cellular functions, it is crucial to maintain the stability of the material in culture. The time-dependent properties of the structures of crosslinked hydrogels can be measured by rheology. The water holding capacity of the

hydrogel is also a highly important feature due to its nature. It is observed that the stiffer hydrogels cause less swelling. Further analysis is done with SEM to examine the hydrogel micro-structure. Since SEM analysis disrupts the natural hydrogel structure, dry samples are obtained by lyophilization before application. Degradation of the hydrogel over time can affect the mechanical and swelling properties and hence cellular behavior. Temperature affects the hydrogel structure critically, low temperatures can generate larger fibril structure, therefore the optimum temperature to be applied in the gelation process should be determined.

Hydrogel formation is essentially carried out by the conversion of liquid precursors into solid substances, with physical (non-covalent or ionic) and chemical (covalent) crosslinking applications.

Physical (ionic) crosslinkage hydrogels, also known as self-assembled hydrogels, include reversible techniques. Physical crosslinking is widely used as it does not involve crosslinking agents that may cause any toxicity risk. Techniques such as freeze-thawing, ionic interactions ($CaCl_2$), hydrogen-bonding, stereocomplex formation and maturation are physical crosslinking applications. However, In physical crosslinking, its flexibility is limited as it has difficulty separating factors such as gelation time, pore size, chemical functionalization and degradation time (47).

Chemical (covalent) crosslinked hydrogels are achieved by establishing covalent bonds between different polymer chains. Chemically crosslinked hydrogels are mechanically more stable and do not easily degrade in solvent due to the presence of covalent bonds. The applications of chemical crosslink are the use of chemical crosslinker agents such as glutaraldehyde, epichlorohydrin, adipicacid dihydrazide and polyaldehydes, chemical and radiation grafting, radical polymerization, high-energy radiation, condensation reaction and enzymatic reaction techniques (56).

2.6. Properties of NK-92 Cells and their Use in Cell Therapy

Natural killer cells, as large granular lymphocytes, provide first-line protection against malignancies, viral infections and against various defects developing in the body by taking part in anti-tumor immune surveillance (79). Although many cell lines are available, particularly the NK-92 cell line is approved by the FDA that can be widely utilized in clinical practices (75). NK-92 was first collected from a 50-year-old male individual with rapidly progressive non-Hodgkin's lymphoma as an NK-like cell line (78).

NK-92, which is highly beneficial for its use in adaptive cell therapy due to its natural existence, can multiply very rapidly ex vivo. Likewise, *in vitro* applications are relatively easy and suitable for various cellular studies. It is a preferred cell line in many cell therapies because of its easy-to-reach needs, its doubling time of 36-48 hours, and its lack of markers to be recognized by other cells.

NK-92 cells are widely applied in cancer therapies as a cytotoxic effector. As in the CAR-T cell therapy developed in recent years, highly successful advancements have been achieved in converting immune cells to therapeutic agents in many cancer therapies, particularly lymphoid malignancies (76). Studies on natural killer cells obtained from peripheral blood, autologously or allogenically, have been on the rise in recent years for their adoption in targeted cancer cell therapy as a safer effector than T cells. Particularly in patients with malignant melanoma, examinations for its use as naive or genetically engineered have become increasingly commonplace (77).

NK-92, which was used in substantial pre-clinical studies, was completed in phase I cancer patients. The first study on 12 patients used NK-92 cells cultured in X-Vivo 10 serum-free medium and pre-irradiated, targeting renal cancer cells. It has been

reported that there are no irreversible side effects and only minor effects occur, and it can be regarded as a new potential in allogeneic cellular immunotherapy applications, specifically in advanced cancer patients (76). In another study, no infusion-related or long-term side effects were observed as a result of NK-92 cells applied to 15 advanced cancer patients (77). However, it was observed that human leukocyte antigen antibodies were produced in one patient, but this case was evaluated as a rare situation for cancer therapy applications of allogenic NK-92.

3. MATERIALS AND METHODS

3.1. Materials

Heat-inactivated Fetal Bovine Serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) (1x) – High Glucose were purchased from Gibco. 2-mercaptoethanol from Gibco, Penicillin-Streptomycin Solution, Trypsin EDTA Solution C x10, Dulbecco's Phosphate Buffered Saline with Calcium and Magnesium (PBS), Dulbecco's Phosphate Buffered Saline without Calcium and Magnesium (PBS w/o), and Trypan Blue Solution from Biological Industries were provided by Acıbadem Labcell. Human Interleukin-2 Recombinant Protein from Sigma-Aldrich was provided by Acıbadem Labcell. X-Vivo™ 10 Serum-Free Medium with L-Glutamine, without Phenol Red and Gentamicin, and X-Vivo™ 15 Serum-Free Medium with L-Glutamine, without Phenol Red and Gentamicin from Lonza Bioscience were provided by Acıbadem Labcell. Hyaluronic acid sodium salt from *Streptococcus equi* and Gelatin from bovine skin were purchased from Sigma-Aldrich. Alginic acid sodium salt from brown algae, Calcium chloride and Glutaraldehyde Solution 50 wt. % in H_2O from Sigma-Aldrich were provided from Nanobiotechnology Center in Acıbadem Mehmet Ali Aydınlar University. 60 mg/mL Dextran 70 9 mg/mL Isotonic Sodium Chloride I.V. Infusion from Polifarma, CryoSure-DMSO from Wak-Chemie Medical GmbH and TexMACS™ GMP Medium from Miltenyi Biotech was provided from Acıbadem Labcell. Standard Carbon Adhesive Tabs, 6 mm dia. 100/pk was purchased from Electron Microscopy Sciences. NK-92 cell line and HEK293T cell line were obtained from Acıbadem Labcell.

3.1.1. Preparation of solutions

1% Alginic Acid Solution

1 mg of Alginic acid sodium salt from brown algae powder was dissolved in 100 mL of distilled water (DW) at a temperature of 40^{o}C on the magnetic stirrer for 10 minutes (mins). Stock solution was stored at +4^{o} C cold room.

4% Alginic Acid Solution

1,6 g of Alginic acid sodium salt from brown algae powder was dissolved in 40 mL of DW at a temperature of 40^{o}C on the magnetic stirrer for 30 mins. Stock solution was stored at +4^{o} C cold room.

1% Gelatin Solution

200 mg of Gelatin from bovine skin powder was dissolved in 20 mL of 1X PBS at a temperature of 40^{o}C on the magnetic stirrer for 20 mins. Stock solution was stored at +4^{o} C cold room.

2% Gelatin Solution

600 mg of Gelatin from bovine skin powder was dissolved in 30 mL of 1X PBS at a temperature of 40^{o}C on the magnetic stirrer for an hour. Stock solution was stored at +4^{o} C cold room.

2% Hyaluronic Acid Solution

800 mg of Hyaluronic acid sodium salt from *S. equi* powder was dissolved in 40 mL of 1X PBS at a temperature of 40^{o}C on the magnetic stirrer for an hour. Stock solution was stored at +4^{o} C cold room.

4% Hyaluronic Acid Solution

800 mg of Hyaluronic acid sodium salt from *S. equi* powder was dissolved in 20 mL of 1X PBS at a temperature of 40^{o}C on the magnetic stirrer for 2 hours. Stock solution was stored at +4^{o} C cold room.

50 mM $CaCl_2$

55,89 mg mass of anhydrous powder with a formula weight of 110.98 was dissolved in 10 mL of DW on the magnetic stirrer for 5 mins. Stock solution was stored at +4^{o} C cold room.

75 mM $CaCl_2$

166,47 mg mass of anhydrous powder with a formula weight of 110.98 was dissolved in 20 mL of DW on the magnetic stirrer for 5 mins. Stock solution was stored at +4^{o} C cold room.

3.2. Methods

3.2.1. Optimization of various hydrogel preparations

In this thesis, hydrogel optimization was studied with three distinct procedures. In the first place, hyaluronic acid and gelatin polymer solutions were mixed with glutaraldehyde (GA) as crosslinker in various ratios and also tested independently as a pure hydrogel. As a second route, alginate polymer solution was added with the mixture of hyaluronic acid and gelatin polymers incorporated with various ratios, and gelation was investigated separately with both glutaraldehyde and calcium chloride ($CaCl_2$) as a crosslinker. The final and third alternative was performed in two experimental groups. Hyaluronic acid concentration and $CaCl_2$ molarity were applied of two different amounts to be performed hydrogel compositions by keeping alginate and gelatin concentrations constant.

3.2.1.1. Hydrogels designed with hyaluronic acid and gelatin polymers applied with glutaraldehyde

Stocks of 2% hyaluronic acid and 1% gelatin polymer solutions stored at +4^{o}C were brought to RT and 50% glutaraldehyde solution stored at RT, was made ready

for use. The procedure for all hydrogels were prepared in glass vials and having a composite volume (vol.) of 900 µl was performed as follows:

- The amounts of gelatin and hyaluronic acid polymers were applied at RT, respectively, as indicated in Table 1. Polymer composite was made by pouring gelatin first and then hyaluronic acid into the vial with a syringe, and vortexed to distribute the components homogeneously.
- Addition of 18 µl and 36 µl of glutaraldehyde separately to 900 µl of composite to be crosslinked was done in a fume hood and vortexed to ensure homogeneous distribution of components (Table 1).
- They were left to gelation for 20-40 mins at 500 rpm on the orbital shaker heated to 40^{o}C.
- 1 mL of DW was added to the vials picked up from the shaker and washed frequently. However, as it will be observed later, DWs were added once and left in the vials, since hydrogels were easily degraded in this process.
- All prepared hydrogels were stored in a cold room at +4^{o}C.
- Wet samples were separated for the rheometer analysis from each hydrogel stored at +4^{o}C, and the remaining vials were left to freeze in the -20^{o}C cold room.
- The samples, which were frozen overnight, were left in the lyophilizer overnight and dry hydrogels were obtained by evaporation of the water in it.
- Dry samples were used in swelling test, SEM and FT-IR analysis.

3.2.1.2. Hydrogels designed with alginate, hyaluronic acid, gelatin polymers applied with glutaraldehyde or calcium chloride

The reason for utilizing the alginate polymer is to eliminate using of glutaraldehyde, which can be toxic in cell culture, to apply a safer crosslinker such as $CaCl_2$ which provides rapid physical (ionic) crosslinking.

Stocks of 1% alginate, 2% hyaluronic acid and 1% gelatin polymer solutions and 50 mM of $CaCl_2$ stored at +4^{o}C were brought to RT and 50% glutaraldehyde solution stored at RT, was made ready for use. All hydrogels with a total volume of 1800 µL were prepared in glass vials.

Table 1. Volumes (µL) and vol. ratios of HA, GEL and GA

2% HA	1% GEL	HA:GEL:GA (9 µL per 450 µL of polymer mixture)	HA:GEL:GA (18 µL per 450 µL of polymer mixture)
1	0	900:0:18	900:0:36
0	1	0:900:18	0:900:36
1	1	450:450:18	450:450:36
1	2	300:600:18	300:600:36
2	1	600:300:18	600:300:36
3	1	675:225:18	675:225:36
1	3	225:675:18	225:675:36

In the first group, the amount of glutaraldehyde to be applied as a crosslinker was 18 µl for each 450 µl composite, and the volumes and ratios of the glutaraldehyde and polymers used are given in Table 2. The process was performed as follows:

- The amounts of gelatin, hyaluronic acid and alginate polymers were applied at RT, respectively, as indicated in Table 2. Polymer composite was prepared by first pouring gelatin, then hyaluronic acid, and finally alginate into the vial with a syringe and vortexed to distribute the components homogeneously.
- Addition of 72 µl of glutaraldehyde separately to 1800 µl of composite to be crosslinked was done in a fume hood and vortexed to ensure homogeneous distribution of components (Table 2).

- They were left to gelation for 20-40 mins at 500 rpm on the orbital shaker heated to 40°C.
- 2 mL of DW was added to the vials picked up from the shaker and washed several times to remove any remaining glutaraldehyde. However, as it will be observed later, DWs were added once and left in the vials, since hydrogels were easily degraded in this process.
- All prepared hydrogels were stored in a cold room at +4°C.
- Wet samples were separated for the rheometer analysis from each hydrogel stored at +4°C, and the remaining vials were left to freeze in the -20°C cold room.
- The samples, which were frozen overnight, were left in the lyophilizer overnight and dry hydrogels were obtained by evaporation of the water in it.
- Dry samples were used in swelling test, SEM and FT-IR analysis.

Table 2. Volumes (µL) and vol. ratios of ALG, HA, GEL and GA

1% ALG	2% HA	1% GEL	ALG:HA:GEL:GA (18 µL per 450 µL of polymer mixture)
1	1	1	600:600:600:72
1	2	1	450:900:450:72
1	1	2	450:450:900:72

In the second group, the amount of $CaCl_2$ to be applied as a crosslinker was used in the same amount as the alginate volume utilized separately in each composite. The hydrogel application formed from polymers with $CaCl_2$, which volumes and ratios are indicated in Table 3, are as follows:

- The amounts of gelatin, hyaluronic acid and alginate polymers were applied, respectively, as indicated in Table 3. Polymer composite was prepared at RT

by first pouring gelatin, then hyaluronic acid, and finally alginate into the vial with a syringe and vortexed to distribute the components homogeneously.

- $CaCl_2$ was applied at RT separately to 1800 µl of composite to be crosslinked and vortexed to ensure homogeneous distribution of the components (Table 3).
- They were left to gelation for 20-40 mins at 500 rpm on the orbital shaker heated to 40°C.
- 2 mL of DW was added to the vials picked up from the shaker and washed several times. However, as it will be observed later, DWs were added once and left in the vials, since hydrogels were easily degraded in this process.
- All prepared hydrogels were stored in a cold room at +4°C.
- Wet samples were separated for the rheometer analysis from each hydrogel stored at +4°C, and the remaining vials were left to freeze in the -20°C cold room.
- The samples, which were frozen overnight, were left in the lyophilizer overnight and dry hydrogels were obtained by evaporation of the water in it.
- Dry samples were used in swelling test, SEM and FT-IR analysis.

Table 3. Volumes (µL) and vol. ratios of ALG, HA, GEL and $CaCl_2$

1% ALG	2% HA	1% GEL	ALG:HA:GEL:$CaCl_2$ (µl)
1	1	0	900:900:0:900
1	0	1	900:0:900:900
1	1	1	600:600:600:600
1	2	1	450:900:450:450
1	1	2	450:450:900:450

3.2.1.3. Hydrogels designed with enhanced concentrations of alginate, hyaluronic acid, gelatin polymers applied with calcium chloride

Alginate, hyaluronic acid and gelatin based hydrogels developed corresponding to the previous test results were performed in two diverse experimental groups. In both groups, hyaluronic acid polymer concentration and $CaCl_2$ molarity were applied in two different amounts in order to improve some properties by keeping alginate and gelatin concentrations constant. The procedure was performed as follows, with a total amount of hydrogel in glass vials of 2 mL:

- Stocks of 4% alginate, 2% hyaluronic acid, 4% hyaluronic acid, 2% gelatin polymer solutions and 50 mM $CaCl_2$ and 75 mM $CaCl_2$ solutions stored at +4^{o}C were brought to RT to be utilized in both groups, separately.
- 2% gelatin, 2% hyaluronic acid and 4% alginate were added into the first vial with syringes in the amounts shown in Table 4, respectively, and vortexed to ensure that the components are homogeneous.
- 75 mM $CaCl_2$ was gently applied onto the polymer mixture and vortexed immediately (Table 4).
- 2% gelatin, 4% hyaluronic acid and 4% alginate were added into the second vial with syringes in the amounts shown in Table 5, respectively, and vortexed to ensure that the components are homogeneous.
- 50 mM $CaCl_2$ was gently applied onto the polymer mixture and vortexed immediately (Table 5).

Table 4. Volumes (µL) of 4% ALG, 2% HA, 2% GEL and 75 mM $CaCl_2$

4% ALG	**2% HA**	**2% GEL**	**75 mM $CaCl_2$**
500 µL	500 µL	500 µL	500 µL

Table 5. Volumes (µL) of 4% ALG, 4% HA, 2% GEL and 50 mM $CaCl_2$

4% ALG	**4% HA**	**2% GEL**	**50 mM $CaCl_2$**
500 µL	500 µL	500 µL	500 µL

- They were both left to gelation for 20-40 mins at 500 rpm on the orbital shaker heated to 40^{o}C.
- 1 mL of DW was added to the vials picked up from the shaker and washed several times. However, as it will be observed later, DWs were added once and left in the vials, since hydrogels were easily degraded in this process.
- Prepared hydrogels was stored at +4^{o}C.
- Wet samples was separated for the rheometer analysis from both hydrogels stored at +4^{o}C, and the remaining samples were left to freeze in the -20^{o}C cold room.
- The samples, which was frozen overnight, were left in the lyophilizer overnight and dry hydrogels were obtained by evaporation of the water in it.
- Dry samples were used in swelling test, SEM and FT-IR analysis.

3.2.2. Optimization of cell cultures

The cell types used in this thesis, NK-92 cell line (2.6), HEK239T cells, and all culture media were obtained from Acıbadem Labcell Cellular Therapy Facility. Particularly since the NK-92 cell line is practiced with various cell culture media in the literature, optimization studies were carried out with certain medium. HEK293T cells were optimized with DMEM only.

HEK293T cells

Many cell lines are derived from parental human embryonic kidney (HEK) 293 cells, an adherent cell type, and these cell lines are involved in the production of enhanced recombinant proteins for the production of therapeutic proteins (72). HEK293T cell line is commonly utilized as a vector for *in vivo* applications (73). This is because HEK293T cells are frequently involved in transfections with a heat-dependent SV40 T antigen, mainly by plasmid expression (74).

It has not been found in the literature to be applied together with hydrogels before, and its use in this thesis is a cell type that is adherent and can proliferate rapidly *in vitro*, purely to serve the purpose of comparison.

3.2.2.1. Preparation of cell culture media

In this thesis, complete DMEM culture medium for HEK293T cells and TexMACS, X-Vivo 10 and X-Vivo 15 complete media, which were prepared exactly the same, for NK-92 cell line were prepared independently. While preparing these complete media, the usage purposes of the supplements were added to the media under the certain conditions are as follows:

- Pen-Strep functions as an antibiotic, preventing bacterial contamination.
- FBS consists of proteins, lipids, and GFs that support cell growth.
- β-mercaptoethanol (1,8 µL per 1 mL) serves as an antioxidant, protecting cells and proteins against oxidative harm.
- IL-2 (1,1 µL per 1 mL) is cytokine for modulating cellular activities.

DMEM (High Glucose) Complete Medium (500 mL)

- 5 mL of Pen-Strep 1% to be used and 50 mL of 10% FBS to be used were thawed in a hot water bath.
- Liquid amounts to be added were first removed from the medium as 55 mL.
- 5 mL of Pen-Strep was added first, and the bottle was shaken.
- 50 mL of FBS was added, and the bottle was shaken again.
- Stock complete medium was stored at +4^{o} C refrigerator.

Table 6. Supplements of DMEM High Glucose complete medium

Total volume of complete medium	**500 mL**
DMEM only	500 mL
1% Pen-Strep	5 mL
10% FBS	50 mL

TexMACS™ Complete Medium (1000 mL)

- 10 mL of Pen-Strep 1% to be used and 100 mL of 10% FBS to be used were thawed in a hot water bath.
- Aliquoted 500 U/mL IL-2 of 1110 µL was allowed to melt at room temperature (RT) for use.
- 1800 µL of β-mercaptoethanol to be used was also taken out of the refrigerator at +4^{o} C to be equal to the RT.
- Liquid amounts to be added were first removed from the medium as 113 mL.
- 10 mL of Pen-Strep, 100 mL of FBS, 1110 µL of IL-2 and 1800 µL of β-mercaptoethanol were added, respectively and the bottle was shaken each addition.

- Stock complete medium was stored at +4^{o} C refrigerator.

Table 7. Supplements of TexMACS complete medium

Total volume of complete medium	**1000 mL**
TexMACS only	1000 mL
1% Pen-Strep	10 mL
10% FBS	100 mL
β-mercaptoethanol	1800 µL
500 U/mL IL-2	1100 µL

X-Vivo™ 10 Complete Medium (1000 mL)

- 10 mL of Pen-Strep 1% to be used and 100 mL of 10% FBS to be used were thawed in a hot water bath.
- Aliquoted 500 U/mL IL-2 of 1110 µL was allowed to melt at RT for use.
- 1800 µL of β-mercaptoethanol to be used was also taken out of the refrigerator at +4^{o} C to be equal to the RT.
- Liquid amounts to be added were first removed from the medium as 113 mL.
- 10 mL of Pen-Strep, 100 mL of FBS, 1110 µL of IL-2 and 1800 µL of β-mercaptoethanol were added, respectively and the bottle was shaken each addition.
- Stock complete medium was stored at +4^{o} C refrigerator.

Table 8. Supplements of X-Vivo 10 complete medium

Total volume of complete medium	**1000 mL**
X-Vivo 10 serum-free medium	1000 mL
1% Pen-Strep	10 mL
10% FBS	100 mL
β-mercaptoethanol	1800 µL
500 U/mL IL-2	1100 µL

X-Vivo™ 15 Complete Medium (1000 mL)

- 10 mL of Pen-Strep 1% to be used and 100 mL of 10% FBS to be used were thawed in a hot water bath.
- Aliquoted 500 U/mL IL-2 of 1110 µL was allowed to melt at RT for use.
- 1800 µL of β-mercaptoethanol to be used was also taken out of the refrigerator at +4^{o} C to be equal to the RT.
- Liquid amounts to be added were first removed from the medium as 113 mL.
- 10 mL of Pen-Strep, 100 mL of FBS, 1110 µL of IL-2 and 1800 µL of β-mercaptoethanol were added, respectively and the bottle was shaken each addition.
- Stock complete medium was stored at +4^{o} C refrigerator.

Table 9. Supplements of X-Vivo 15 complete medium

Total volume of complete medium	**1000 mL**
X-Vivo 15 serum-free medium	1000 mL
1% Pen-Strep	10 mL
10% FBS	100 mL
β-mercaptoethanol	1800 µL
500 U/mL IL-2	1100 µL

3.2.3. Optimization of NK-92 cell culture conditions

In order to optimize the NK-92 cell culture, which is an IL-2 dependent cell line, studies were performed with TexMACS, X-Vivo 10 and X-Vivo 15 cell media. Before the optimization, 5 mL of cryovial from Acıbadem Labcell was dissolved in a hot water bath. The method for frozen cells after thawing proceeds as follows:

- The cryovial was washed with PBS w/o in case of any cells remaining in it, and transferred to the 15 mL falcon tube and diluted with 1X PBS w/o to 10 mL.
- It was centrifuged at 200 G and 10 mins.
- After centrifugation, the supernatant was removed, and the pellet was diluted to 10 mL with PBS with Ca^{+2} and Mg^{+2} and homogenized by pipetting.
- It was centrifuged at 200 G and 10 mins for the second time.
- After centrifugation, the supernatant was removed, and since the medium used before the cryopreservation is TexMACS, it was diluted to 5 mL with TexMACS and the pellet was homogenized by pipetting.
- Subsequently, cell number and viability were measured in the automated cell counter device with trypan blue to determine the flask size to be seeded.

Cells were divided into three equal groups after cell counting. One of the three distinct culture flasks was loaded with complete TexMACS prepared as shown in Table 7, one with complete X-Vivo 10 medium prepared as shown in Table 8, and one with complete X-Vivo 15 medium prepared as shown in Table 9. Cells were seeded into these three flasks separately. Cells were incubated in a CO_2 incubator at 37^{o}C for 48-72 hours.

After 48-72 hours of incubation, the cells were observed under the light microscope and their proliferation was verified. Subsequently, they were placed in falcon tubes and centrifuged at 200 G for 10 minutes and the supernatant was removed after centrifugation. Dilution of cells at each cell passaging after this step was visually dependent on their density, ranging from 5 to 15 mL. Cell counting was performed using trypan blue with cells taken from the pellet diluted as detailed above.

Since the doubling time of NK-92, which is a constantly expanding cell line, is between 2-4 days, a lot of cells were obtained in each passage and some of these cells were cultivated over and some of them were cryopreserved. Cryopreservation was carried out as adding 4 mL of freezing solution for 1 mL of cells was mixed in 5 mL cryovials. The freezing solution was obtained from a 3/2 ratio of dextran/DMSO mixture. The most critical point to note here is that the cryovials should be positioned on ice racks kept in the -20^{o}C while adding the freezing solution to the cells. Cryovials should be put in the freezer at -80^{o}C without wasting time.

The media were changed every 2-3 days with this procedure, and the passage of cells was repeated an average of 12-14 times. The viability data obtained in cell counts after each passage with sets used complete TexMACS, complete X-Vivo 10 and complete X-Vivo 15 are shown in Figures 2.1, 2.2 and 2.3, respectively.

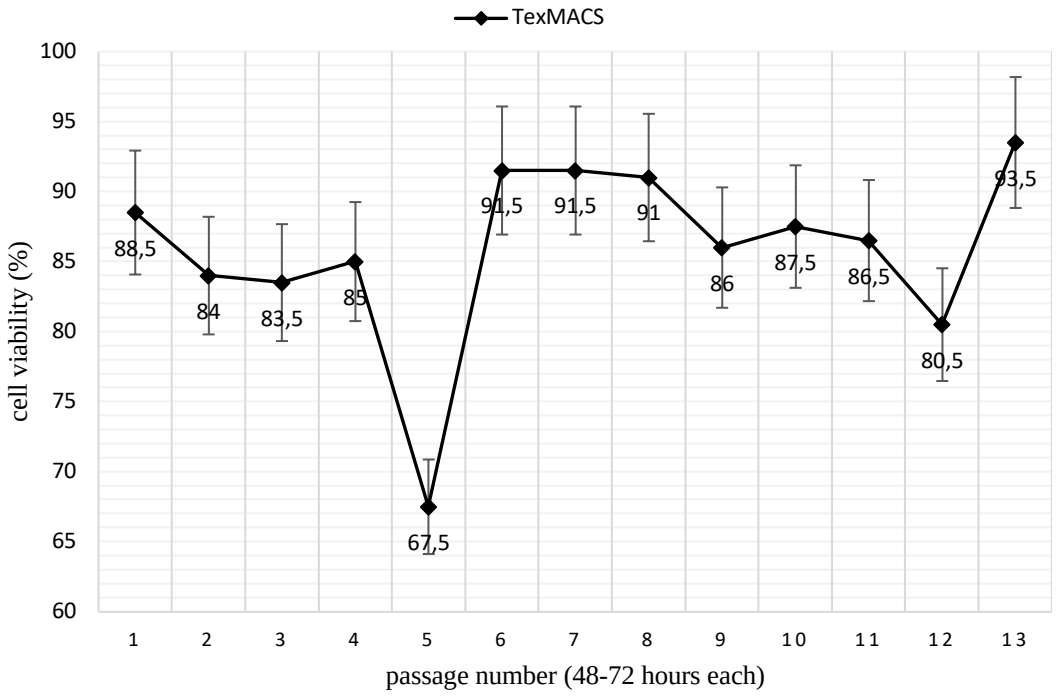

Figure 2.1. Cell viability percentages in TexMACS complete medium

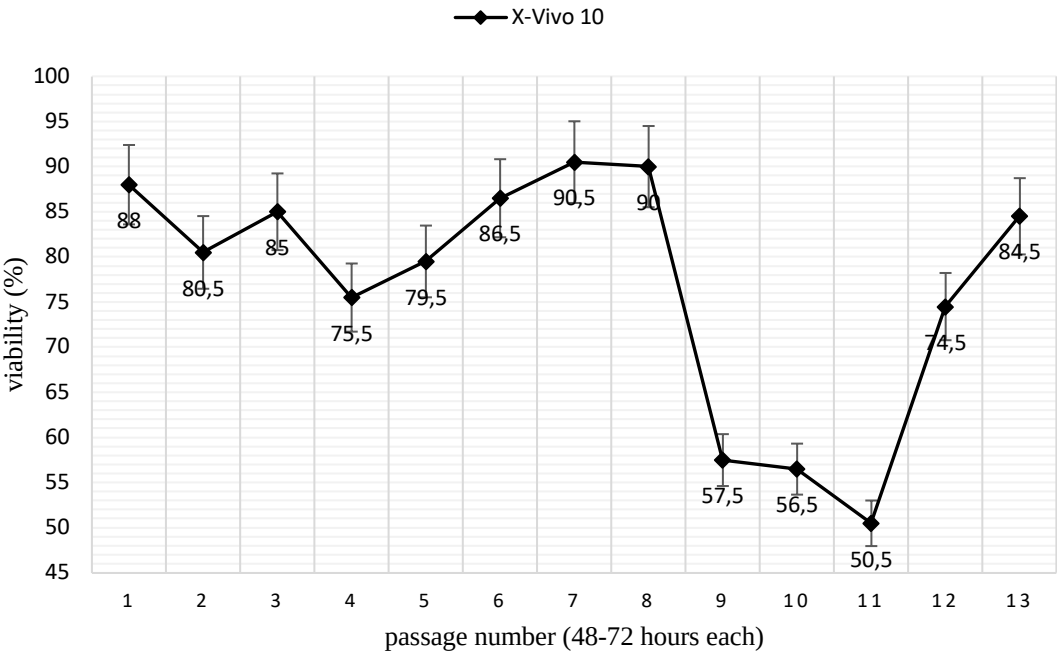

Figure 2.2. Cell viability percentages in X-Vivo 10 complete medium

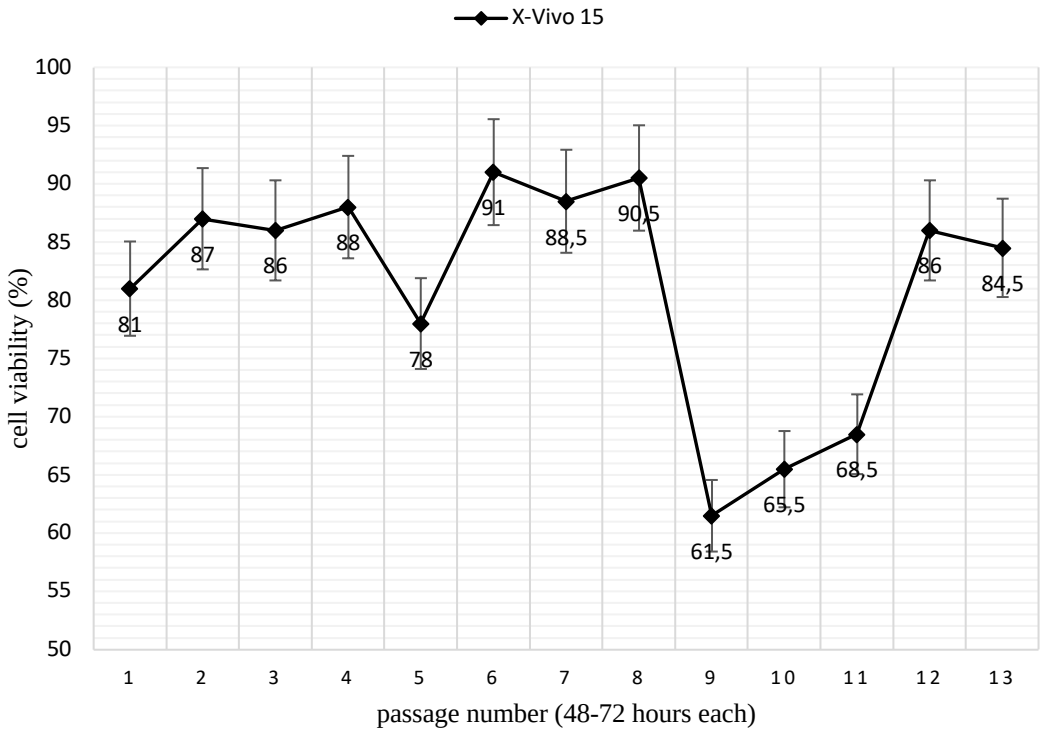

Figure 2.3. Cell viability percentages in X-Vivo 15 complete medium

3.2.4. *In vitro* applications

As a result of hydrogel preparations and cell culture optimizations obtained through various experiments, 4% alginate, 2% hyaluronic acid, 2% gelatin with 75 mM $CaCl_2$ and 4% alginate, 4% hyaluronic acid, 2% gelatin with 50 mM $CaCl_2$ hydrogel designs were studied in duplicate and performed in 24-well-plates. An artificial 3D scaffold system was achieved by mixing the NK-92 cell line with X-Vivo 15 culture medium and HEK293T cells with DMEM separately with polymers during the gelation process of these two hydrogel preparations. The application was carried out as adding gelatin, cells, hyaluronic acid, alginate and $CaCl_2$, respectively. This order, in which polymers and cells were added, was determined in a manner that cells could adapt most in cell-laden hydrogel application. The purpose of putting the crosslinker last is that it makes the hydrogel stiff as soon as $CaCl_2$ is added.

Since the hydrogel composite is typically vortexed at each substance addition step, one of the duplicate samples was mixed in the vial and vortexed, while the other sample was mixed directly in the well and homogenized by gentle shaking by hand. They were obtained with this procedure to investigate whether vortexing will have an effect on the cells while the cell-laden hydrogel system was being set up.

3.2.4.1. Preparation of cell-laden hydrogels

Both hydrogels were prepared as duplicate sets in 24-well-plates, one by vortexing each time substance is added, and the other one by gently shaking the well-plate to ensure homogeneity.

In the first pair of wells, 200 µL of 2% gelatin, 500 µL of NK-92 cells were counted as a million, 200 µL of 2% hyaluronic acid, 200 µL of 4% alginate were placed in the Eppendorf tube and the other in the well, respectively. The plate was shaken gently and the Eppendorf tube was vortexed for 2 seconds to ensure homogeneity. After the composite in the Eppendorf tube was poured into the well, 200 µL of 75 mM $CaCl_2$ as crosslinker was placed on both wells to be given dropwise with a micropipette.

In the second pair of wells, 200 µL of 2% gelatin, 500 µL of NK-92 cells were counted as a million, 200 µL of 4% hyaluronic acid, 200 µL of 4% alginate were placed in the Eppendorf tube and the other in the well, respectively. The plate was shaken gently and the Eppendorf tube was vortexed for 2 seconds to ensure homogeneity. After the composite in the Eppendorf tube was poured into the well, 200 µL of 50 mM $CaCl_2$ as crosslinker was placed on both wells to be given dropwise with a micropipette.

After both groups were incubated at 37°C in CO_2 incubator for two hours, 200 µL of complete X-Vivo 15 was added to each well and placed back into the CO_2 incubator for 48-72 hours incubation.

In the third pair of wells, 200 µL of 2% gelatin, 250 µL of HEK293T cells were counted as a million, 200 µL of 2% hyaluronic acid, 200 µL of 4% alginate were placed in the Eppendorf tube and the other in the well, respectively. The plate was shaken gently and the Eppendorf tube was vortexed for 2 seconds to ensure homogeneity. After the composite in the Eppendorf tube was poured into the well, 200 µL of 75 mM $CaCl_2$ as crosslinker was placed on both wells to be given dropwise with a micropipette.

In the last pair of wells, 200 µL of 2% gelatin, 250 µL of HEK293T cells were counted as a million, 200 µL of 4% hyaluronic acid, 200 µL of 4% alginate were placed in the Eppendorf tube and the other in the well, respectively. The plate was shaken gently and the Eppendorf tube was vortexed for 2 seconds to ensure homogeneity. After the composite in the Eppendorf tube was poured into the well, 200 µL of 50 mM $CaCl_2$ as crosslinker was placed on both wells to be given dropwise with a micropipette.

After both groups were incubated at 37°C in CO_2 incubator for two hours, 200 µL of complete DMEM was added to each well and placed back into the CO_2 incubator for 48-72 hours incubation.

Fixation was performed after 48-72 hours by taking samples from cell-laden hydrogels for histological analysis.

3.2.5. Trypan blue exclusion test of cell viability

100 µL of cell population from the diluted pellet was mixed with 100 µL of trypan blue. 10 µL from the mix was put into counting slides. Cells were counted by TC20™ Automated Cell Counter (Bio-Rad Laboratories, Inc.). The counter showed the number of dead and live cells. Viability was calculated by division of live cell number with total cell number.

3.2.6. Statistical analysis

All the samples were analyzed by using Student's *t*-test. The results were represented as the mean ± SD of at least 3 experimental repeats. P value < 0.05 was considered as statistically significant.

4. RESULTS

4.1. Optimization Assessment of NK-92 Cell Culture Conditions

Three different serum-free media, TexMACS, X-Vivo 10 and X-Vivo 15, were utilized for the optimization of NK-92 cell culture, which is a continuously expanding cell line. After the complete media were prepared by determining the supplements and their amounts corresponding to the requirements of NK-92, the cell culture studies continued for an average of 12-14 passages. The viability of cells seeded in TexMACS, X-Vivo 10 and X-Vivo 15 complete media was compared.

The average viability of cells seeded in TexMACS complete medium was calculated as 85.9%. According to the chart in Figure 2.1, the reason for the decrease in cell viability around 5th passage is thought to be related to environmental conditions since it has no contamination occurs. Accordingly, TexMACS culture medium is considered to be a highly sufficient choice for providing the viability and proliferation of the NK-92 cell line.

The viability of cells seeded in X-Vivo 10 complete medium was calculated as 76.8% on average. According to the chart in Figure 2.2, it was observed that the cell viability decreased between the 9th and 11th passages. The reason for the decrease may be related to environmental conditions, because no contamination has occurred. As a matter of fact, it was noticed that the percentage of viability increased afterwards. Even if it is not considered as the first alternative, it is considered that it can be utilized to maintain the viability and proliferation of the NK-92 cell line.

Subsequently, the viability of cells seeded in X-Vivo 15 complete medium was calculated as 81.2% on average. According to the chart in Figure 2.3, it was observed that the viability of the cells decreased between the 9th and 11th passages, similar to the practice of X-Vivo 10 culture medium. However, when compared to X-Vivo 10, it is seen that the amount of decrease is less. The reason for the decrease may also be related to environmental conditions because no contamination has occurred. Similarly, it is noticed that the percentage of viability increased again. X-Vivo 15 culture medium is considered to be a choice that can be utilized to maintain the viability and proliferation of the NK-92 cell line.

TexMACS complete medium is considered to be the most suitable option, as it has been observed that it provides better stability among these three complete media in order to support the viability and proliferation of the NK-92 cell line. Compared to X-Vivo 10, X-Vivo 15 complete medium is considered to be the second applicable option due to the higher percentage of viability and better stability of the cells.

As a result of these evaluations, it is observed that the ideal culture medium for the NK-92 cell line is TexMACS. However, although these three cell culture media were supplied from Acıbadem Labcell, X-Vivo 15 culture medium was utilized in cell-laden hydrogel preparation studies due to the issues in the supply of TexMACS culture medium regardless of the results evaluated above.

4.2. Evaluation of Hydrogels Designed with Various Polymers

The swelling test, SEM and FT-IR spectroscopy analyzes were studied to evaluate the designed hydrogels were performed on dry samples. In order to obtain dry samples, freeze drying, also known as lyophilization, was applied to hydrogels. For this process, the formed samples were left in DW added after gelation and allowed to freeze in the

-20°C cold room overnight. Many pits were pierced with a needle thus water molecules could escape from the caps of the vials where the samples were placed. The frozen samples were left in the lyophilizer overnight and the water molecules were sublimated in the hydrogels. This sublimation facilitates the formation of a hydrogel with a porous structure as desired. Rheological analysis was examined on wet samples of hydrogels. Therefore, after preparing the samples, storing them at +4°C is sufficient to apply the rheological analysis at any time.

In this part, hydrogels were achieved by utilizing in various volumes of 2% hyaluronic acid and 1% gelatin polymers crosslinked with glutaraldehyde, which is the first group of hydrogel designs, will be investigated first. The data obtained as a result of the studies indicated in Table 1 were evaluated. In the initial phase, without any further analysis, the HA:GEL volume ratios of 1:3 and the pure GEL remained completely liquid during the gelation process and could not form a hydrogel.

In the second group analysis, hydrogels were achieved by utilizing of 1% alginate, 2% hyaluronic acid and 1% gelatin polymers in various volumes as shown in Table 2 and Table 3, and crosslinked separately with glutaraldehyde and $CaCl_2$, were investigated.

In the third and final group, the concentrations of 4% alginate and 2% gelatin polymers were maintained constant and the concentration of hyaluronic acid polymer was utilized as 2% and 4%, respectively. The hydrogels were achieved by crosslinking these polymer mixtures with 75 mM and 50 mM $CaCl_2$, respectively, were investigated.

4.2.1. Swelling test results

The swelling test involves the interaction between the matrix and the solvent and is the major indicator of whether the material obtained is hydrogel. Subsequently, the hydrogel should not be a solvent-soluble material, but a solvent-swallowed material. Dry samples were obtained by putting the samples in a lyophilizer overnight before applying the swelling test. Corresponding to the amounts of dry samples, determined volume of solvents was added to the dry hydrogels each time and stayed for 5 minutes. After 5 minutes, the solvent was drawn with a micropipette and the wet weights of the samples were measured. This process continues until the hydrogel disperses and loses mass. The swelling test is usually performed with the solvent utilized to achieve the polymer solutions used in the hydrogel. To calculate the swelling ratio, the amount of solvent absorbed per unit time was measured and the following formula was used:

$$\text{Swelling ratio (\%)} = \frac{W_s - W_{d.}}{W_d} \text{ X } 100$$

(W_d: Weight of dry hydrogel, W_s: Weight of swollen hydrogel)

In the first experimental model, 2% hyaluronic acid and 1% gelatin polymers were utilized to produce hydrogels with glutaraldehyde as crosslinker, with volume ratios of HA:GEL 1:1, 1:2, 2:1, 3:1 and pure HA. As a result of the swelling test performed with DW, all hydrogel samples could not remain stable in DW from the first trial and dispersed.

In the second experimental model, hydrogels were achieved by utilizing 1% alginate, 2% hyaluronic acid and 1% gelatin together with glutaraldehyde and $CaCl_2$ separately as crosslinkers. Hydrogels using glutaraldehyde as a crosslinker were

produced with ALG:HA:GEL volume ratios of 1:1:1, 1:2:1 and 1:1:2. As a result of the swelling test performed with DW, all hydrogel samples could not remain stable in DW from the first trial and dispersed. Therefore, the swelling test was renewed a second time with PBS. The reason for applying PBS is that ALG polymer and $CaCl_2$ solution were prepared with PBS. The samples did not remain stable as desired in the second time and were dispersed directly in PBS.

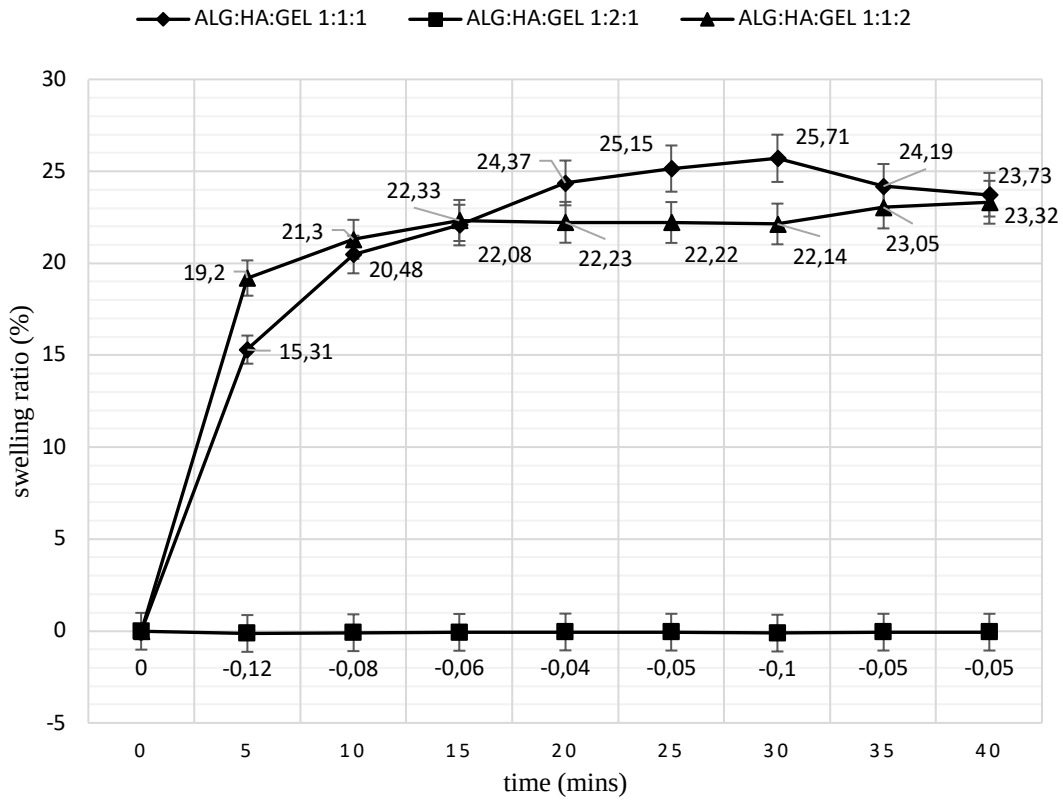

Figure 3.1. Swelling ratios (%) of 1%ALG:2%HA:1%GEL w/ $CaCl_2$

Hydrogels by utilizing 1% alginate, 2% hyaluronic acid and 1% gelatin polymers together with $CaCl_2$ as crosslinker in various volume ratios ALG:HA:GEL as 1:1:1, 1:2:1 and 1:1: 2 have been obtained. When the swelling test was applied to the samples with PBS, it was observed that they did not disperse as quickly as the samples crosslinked with glutaraldehyde in the solvent. The swelling ratio chart of the samples

that can remain stable in the solvent for a longer time is shown in Figure 3.1. Since it can be observed in the chart with the swelling ratio values, it was declared that the swelling properties of ALG:HA:GEL 1:1:1 and 1:1:2 variations progressed partly in a stable form, however, the 1:2:1 variation lost mass from the first trial. In addition to these results, swelling test using PBS was applied to the hydrogels of ALG:HA (1:1) and ALG:GEL (1:1) which were achieved by crosslinking with $CaCl_2$, the amounts utilized in the mixture are shown in Table 3. As a result, it was observed that the ALG:GEL hydrogel did not remain stable and dispersed in any situation in PBS, while the ALG:HA hydrogel did not directly disperse in PBS, but lost mass by degrading each time.

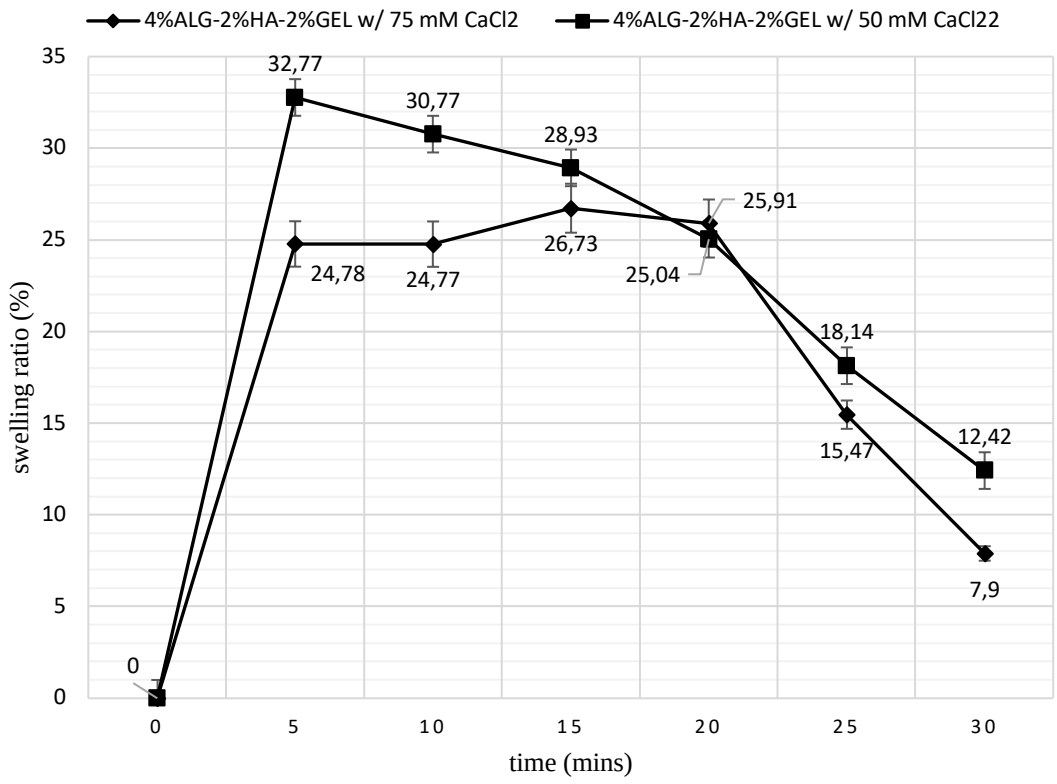

Figure 3.2. Swelling ratios (%) of ALG:HA:GEL w/ $CaCl_2$ with enhanced conc.

As the third hydrogel preparation, the hydrogels were obtained with 2 different variations were tested for swelling using PBS. The hydrogel was achieved by utilizing 4% alginate, 2% hyaluronic acid and 2% gelatin polymers together with 75 mM $CaCl_2$ as a crosslinker in equal volume as shown in Table 4 in the first group was dried by keeping it in a lyophilizer overnight. In the second group, the hydrogel was achieved by utilizing 4% alginate, 4% hyaluronic acid and 2% gelatin polymers together with 50 mM $CaCl_2$ as a crosslinker in equal volume as shown in Table 5, was dried by keeping it in a lyophilizer overnight. Each time 2 mL of PBS was added to the obtained dry samples, stayed for 5 minutes and then drawn with a micropipette. Both hydrogels were observed to be more stable than early examinations (Figure 3.2).

4.2.2. Scanning electron microscope (SEM) analysis

Hydrogels were achieved by utilizing various concentrations and volume ratios of alginate, hyaluronic acid and gelatin polymers were investigated in SEM for morphology analysis. Scanning electron microscope (SEM) analyzes were performed with Quattro ESEM (The Thermo Scientific^{TM}). In order to make proper visualization in SEM, dry samples were handled in this examination. The required amount of sections were taken from the samples that became dry with the lyophilizer, and they were placed on the adhesive carbon tabs. The samples put in the SEM were investigated under both high vacuum (coating) and low vacuum (LV) at various angles and magnifications, and appropriate images were captured. Low vacuum is commonly used in low magnification examinations. On the other hand, in the coating technique, the samples were sputter-coated with gold. This technique is often used on hydrated samples. The pore sizes were calculated and indicated in the obtained images.

The SEM analysis of hydrogels obtained from 2% hyaluronic acid and 1% gelatin polymers were achieved by applying glutaraldehyde as crosslink in various volumes of ALG:GEL 1:1, 1:2, 2:1, 3:1 and pure HA are as follows:

- No suitable porous structures were formed in the pure HA hydrogel (Figure 4.1).
- When the morphology of the hydrogel obtained with HA:GEL volume ratios of 1:1 was examined, porous structures that were considered to be insufficient were observed (Figure 4.2).
- When the morphology of the hydrogel obtained with HA:GEL volume ratios of 1:2 was examined, a smooth surface and heterogeneously distributed porous structure was observed which is not considered to be sufficient (Figure 4.3).
- When the morphology of the hydrogel obtained with HA:GEL volume ratios of 2:1 was examined, it was observed that the gelatin molecules crosslinked within themselves and constituted a speckled surface (Figure 4.4). Also pore sizes were relatively large for the cell types used in this thesis.
- When the morphology of the hydrogel obtained with HA:GEL volume ratios of 3:1 was examined, crosslinking did not occur as much as needed due to the high amount of hyaluronic acid (Figure 4.5).

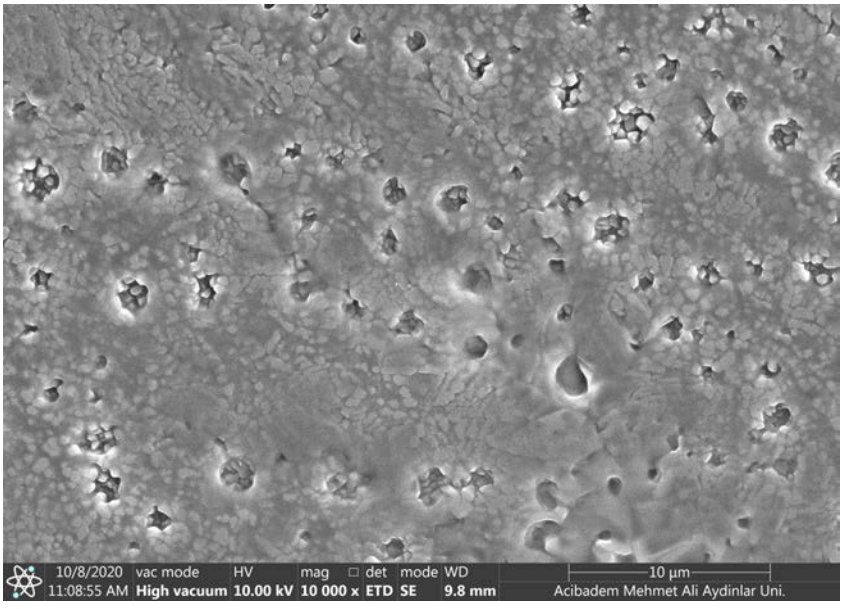

Figure 4.1. SEM image of pure HA w/ GA hydrogel

The samples was examined with coating. It is observed that pure HA hydrogel is not suitable for attachment of cells.

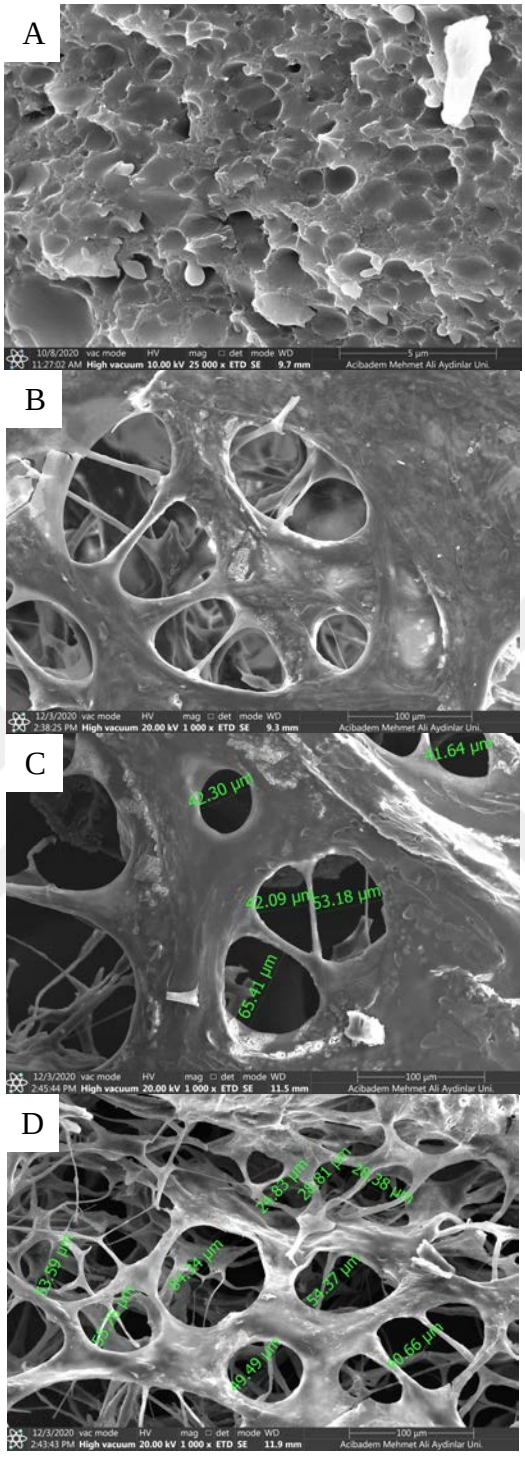

Figure 4.2. SEM images of HA:GEL 1:1 w/ GA hydrogel

The samples was examined with coating**.** It is observed that the pore formation is not developed as desired. Pores are observed that are not homogeneously distributed (A) and have a tear-like shape (B), which are considered to be unsuitable for the attachment of cells. The pore sizes are shown with 1000X magnification (C, D).

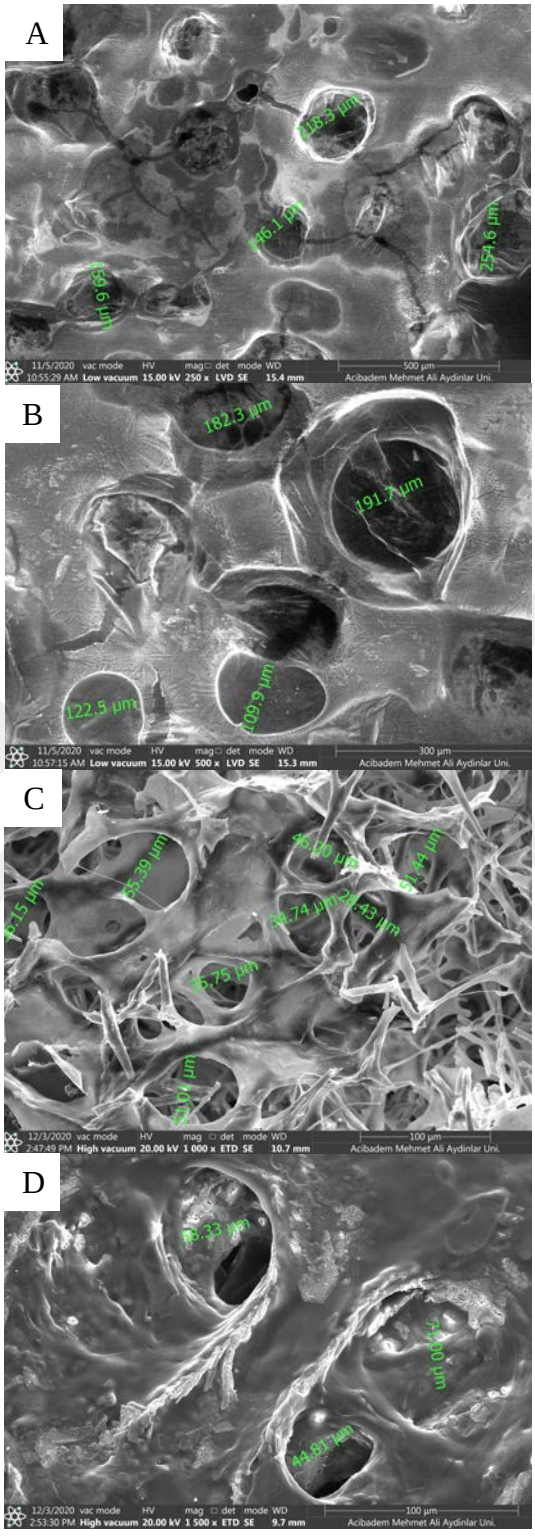

Figure 4.3. SEM images of HA:GEL 1:2 w/ GA hydrogel

The samples were examined both under LV and with coating**.** Smooth surface is observed due to the presence of HA polymer. The pore sizes are shown with 250X magnification(A), 500X magnification (B), 1000X magnification (C) and 1500X magnification (D).

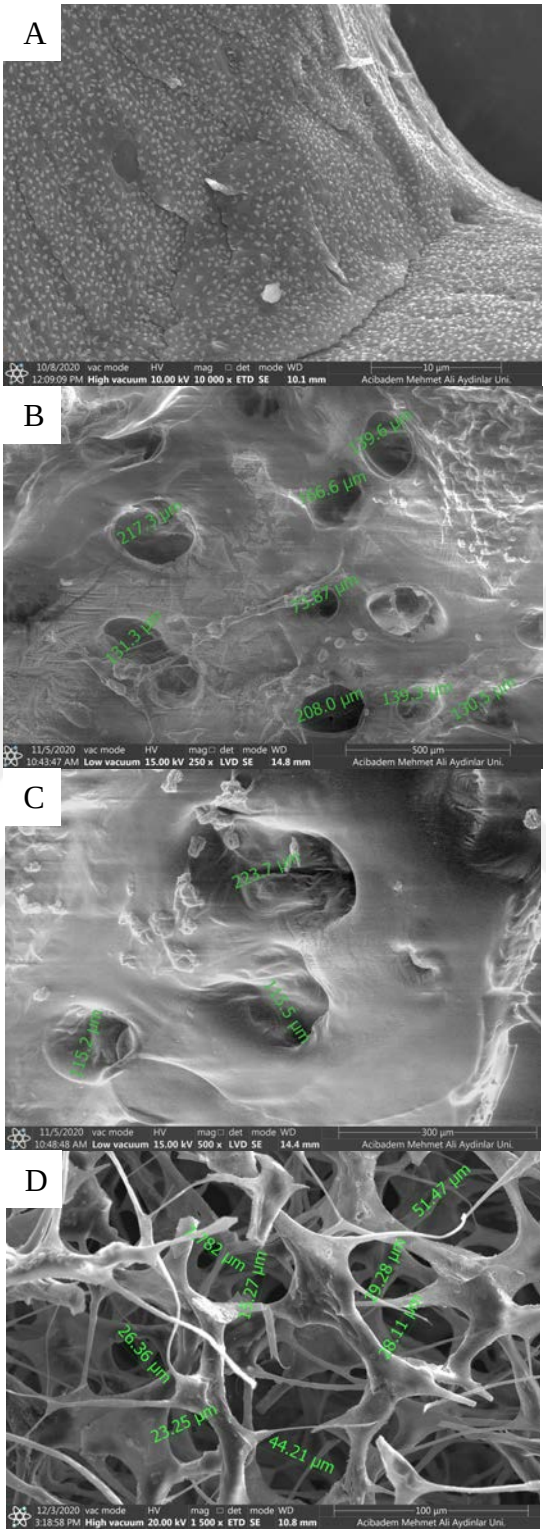

Figure 4.4. SEM images of HA:GEL 2:1 w/ GA hydrogel.

The samples were examined both under LV and with coating**.** Speckled surface can be seen occurred with GEL molecules crosslinked within themselves (A). The pore sizes are shown with 250X magnification (B), 500X magnification (C), and 1500X magnification (D).

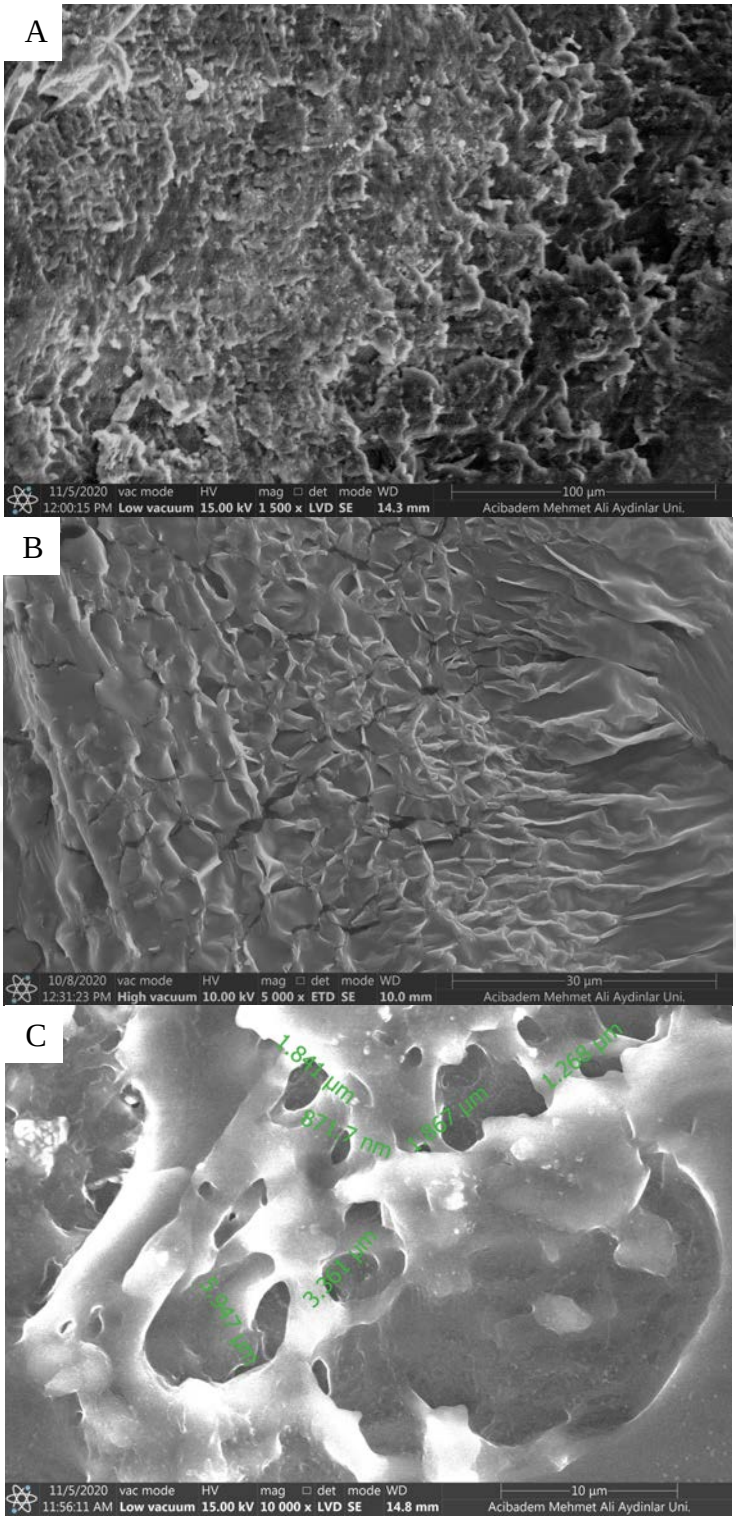

Figure 4.5. SEM images of HA:GEL 3:1 w/ GA hydrogel.

The samples were examined both under LV and with coating**.** It is observed that crosslinking was not occurred sufficient due to the amount of HA polymer and porous structure was not obtained (A, B). The insufficient pore sizes are shown with 10000X magnification (C).

The SEM analysis of hydrogels obtained from 1% alginate, 2% hyaluronic acid and 1% gelatin polymers were achieved by applying glutaraldehyde as crosslink in various volumes of ALG:HA:GEL 1:1:1, 1:2:1, and 1:1:2 are as follows:

- In the hydrogel design which the volume ratio of ALG:HA:GEL was obtained as 1:1:1 and crosslinked with glutaraldehyde, crosslinking was observed to be good and it is considered that the pore sizes may be suitable for the placement of cells (Figure 5.1).
- In the hydrogel which the volume ratio of ALG:HA:GEL was obtained as 1:2:1 and crosslinked with glutaraldehyde, it was observed that the crosslinking did not occur well since the concentration and amount of hyaluronic acid polymer was higher than the other two polymers. It is considered that the formed pores may not be sufficient for the attachment of cells (Figure 5.2).
- In the hydrogel which ALG:HA:GEL volume ratios were obtained as 1:1:2 and crosslinked with glutaraldehyde, it was observed that the crosslinking did not occur successfully and the uncrosslinked parts caused accumulation on the surface due to the high amount of gelatin polymer. Although it is assumed that the pore sizes may be sufficient for cells, it is considered that they may not be suitable for their attachment (Figure 5.3).

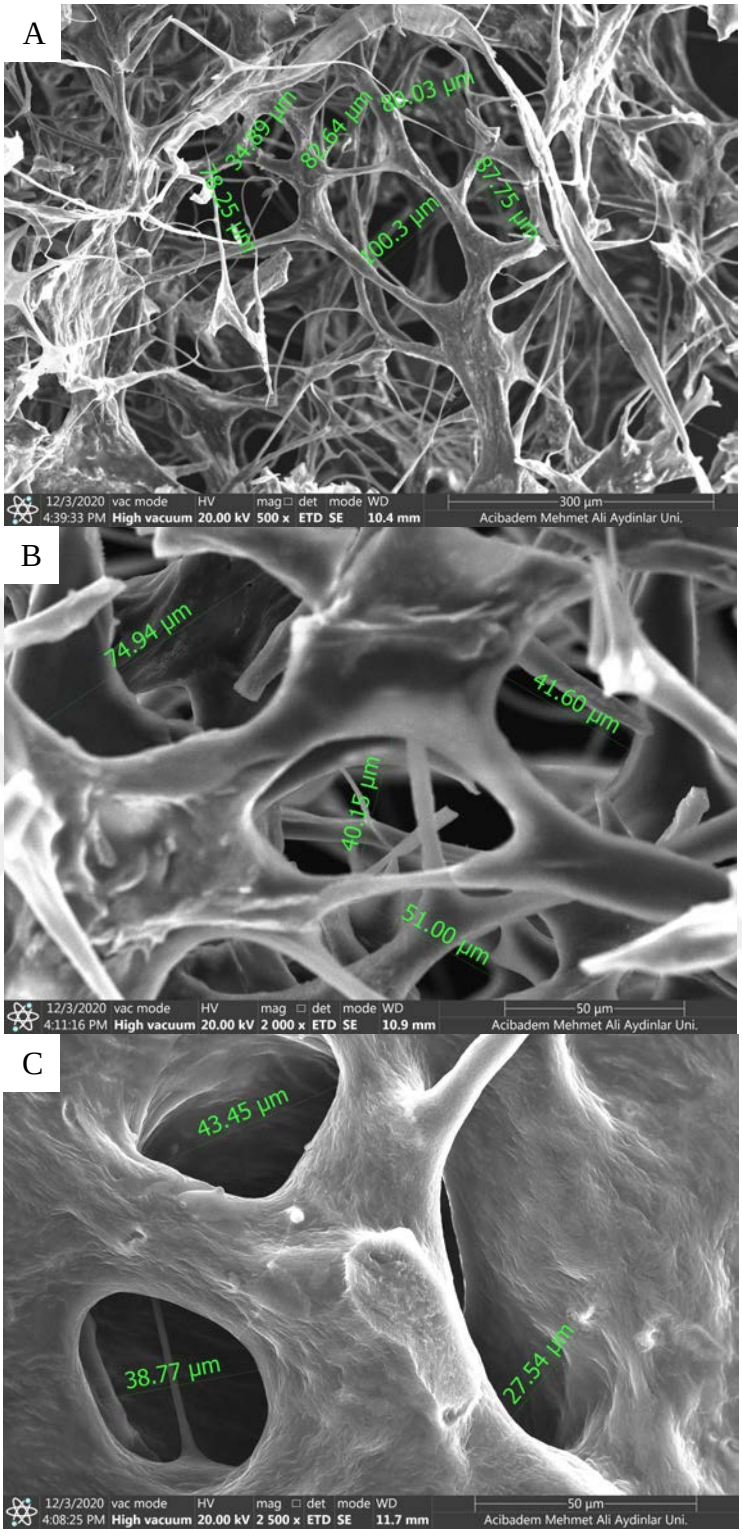

Figure 5.1. SEM images of ALG:HA:GEL 1:1:1 w/ GA hydrogel.

The samples were examined with coating**.** The pore sizes are shown with 500X magnification (A), 2000X magnification (B) and 2500X magnification (C).

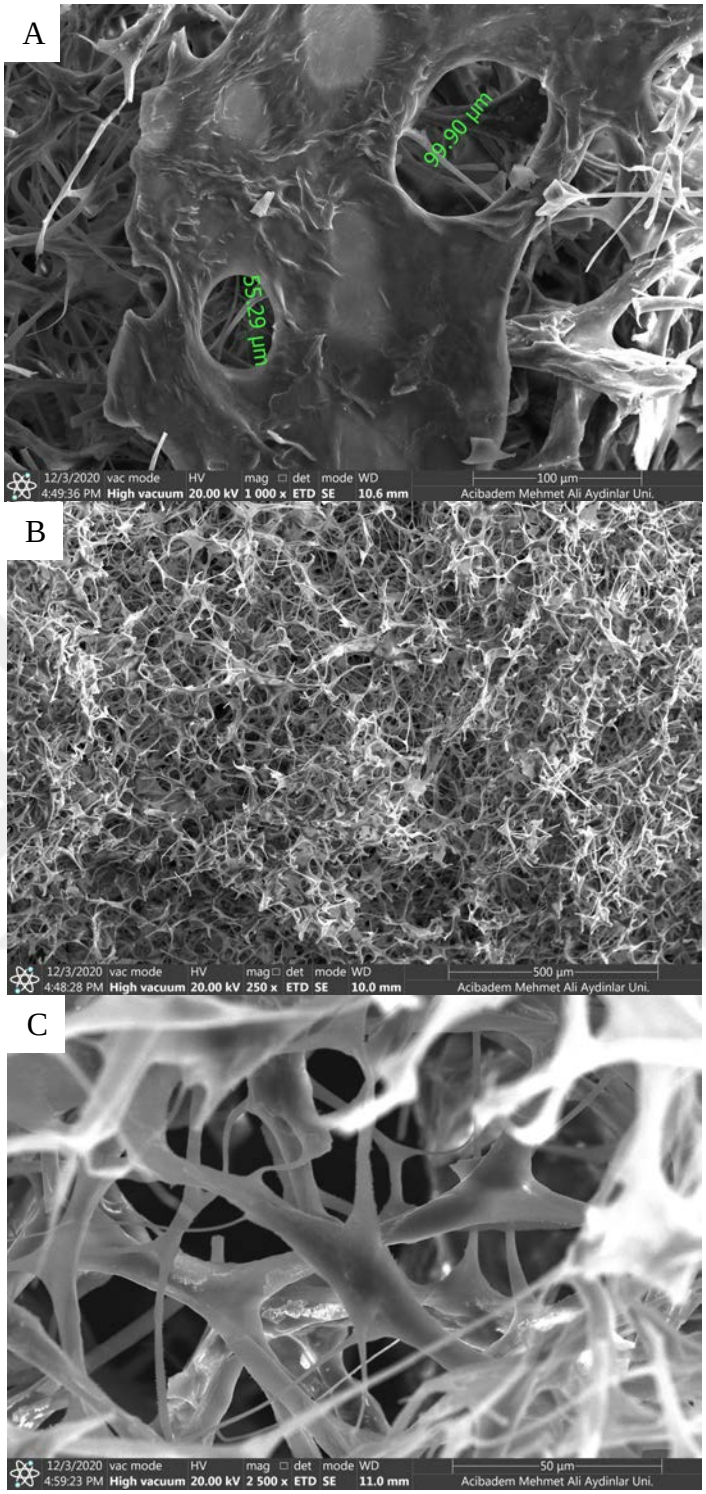

Figure 5.2. SEM images of ALG:HA:GEL 1:2:1 w/ GA hydrogel.

The samples were examined with coating. The pore sizes are shown with 1000X magnification (A). Unsuitable porous structure of hydrogel is observed with 250X magnification (B) and 2500X magnification (C).

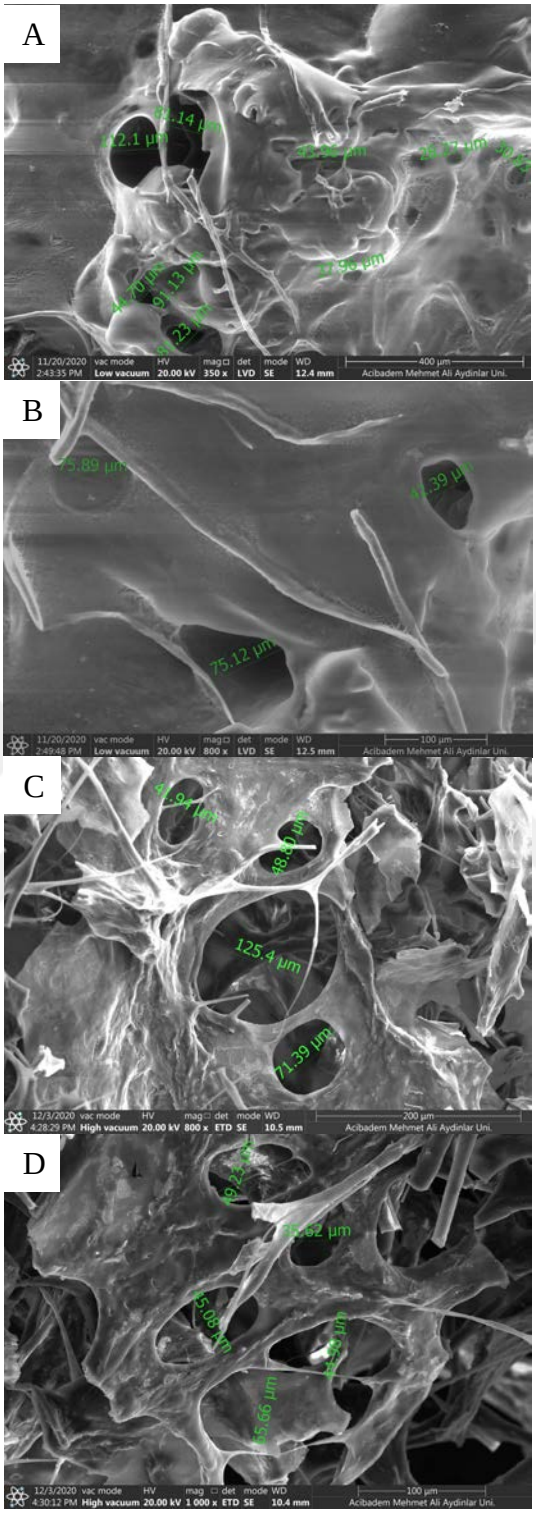

Figure 5.3. SEM images of ALG:HA:GEL 1:1:2 w/ GA hydrogel.

The samples were examined both under LV and with coating**.** The insufficient pore sizes are shown with 350X magnification (A), 800X magnification (B, C) and 1000X magnification (D).

The SEM analysis of hydrogels obtained from 1% alginate, 2% hyaluronic acid and 1% gelatin polymers were achieved by applying $CaCl_2$ as crosslink in various volumes of ALG:HA:GEL 1:1:1, 1:2:1, and 1:1:2 are as follows:

- In the hydrogel design, where the volumes of ALG:HA:GEL polymers were utilized as 1:1:1 and obtained by crosslinking with $CaCl_2$, porous structures that are thought to be suitable for cell attachment were observed. However, since the pore sizes are very extensive, it is considered not suitable for the cell types used in this thesis.
- In the hydrogel design, where the volumes of ALG:HA:GEL polymers were utilized as 1:2:1 and obtained by crosslinking with $CaCl_2$, the crosslinking could not develop sufficiently because the hyaluronic acid polymer concentration and amount are higher than the other two polymers. In addition, pore sizes are considered to be very extensive for the placement of cells.
- It is considered that the hydrogel design was achieved by crosslinking with $CaCl_2$, where the volumes of ALG:HA:GEL polymers were utilized as 1:1:2, is not a sufficient model because the pore sizes are very extensive and there is no homogeneous pore structure suitable for the placement of cells. In addition, it is observed that crosslinking does not occur as desired due to the high amount of gelatin polymer.

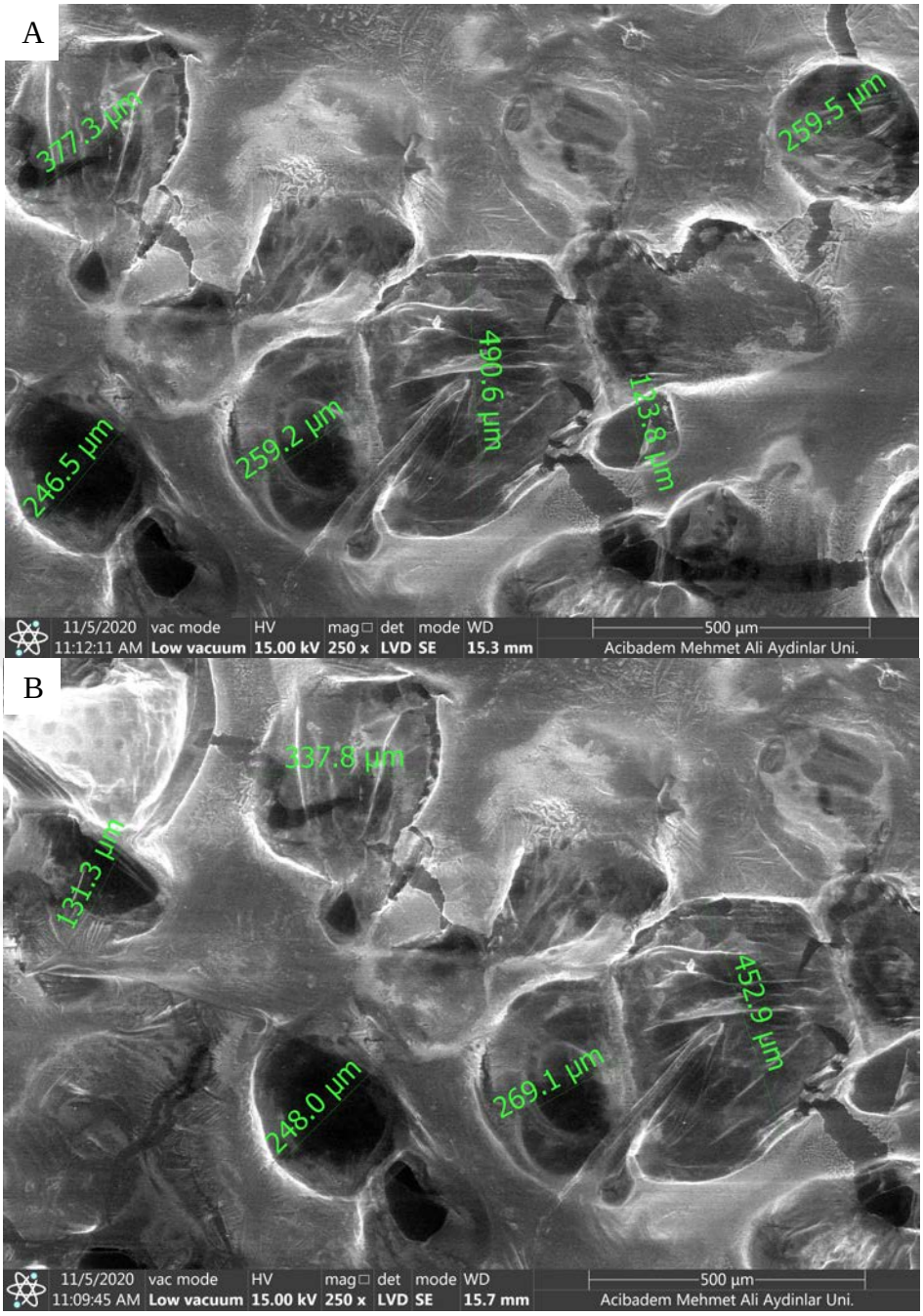

Figure 6.1. SEM images of ALG:HA:GEL 1:1:1 w/ $CaCl_2$ hydrogel.

The samples were examined under low vacuum. The pore sizes, which very extensive, are shown with 250X magnification (A, B).

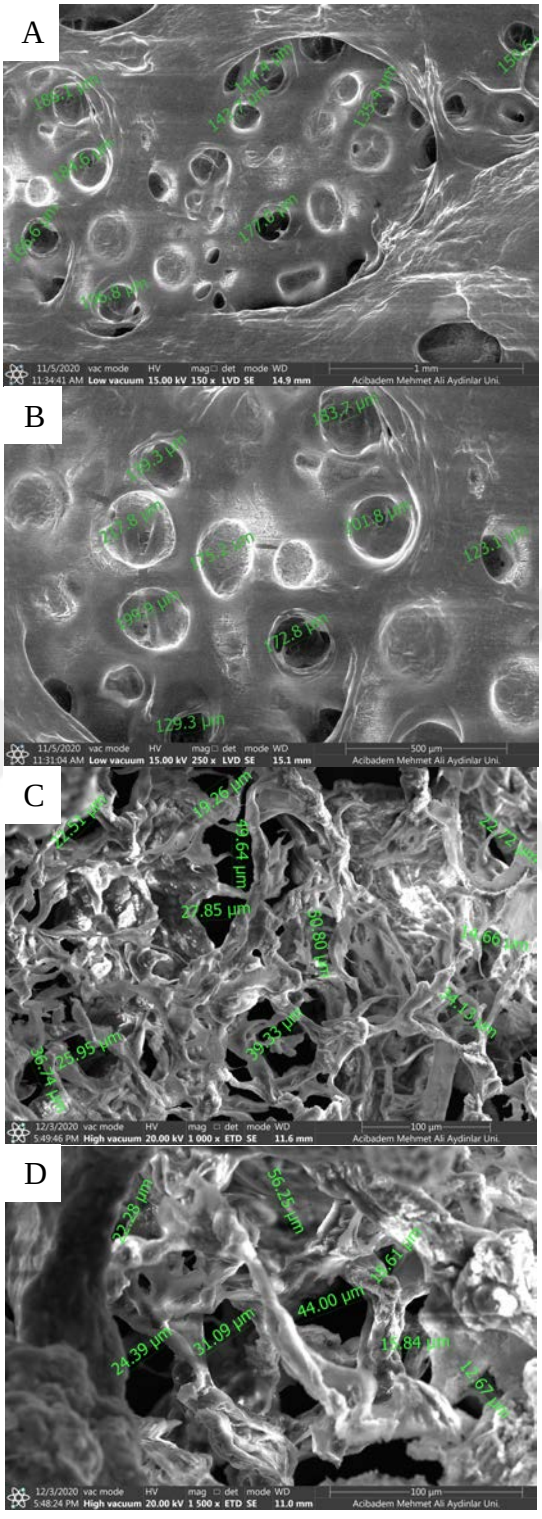

Figure 6.2. SEM images of ALG:HA:GEL 1:2:1 w/ $CaCl_2$ hydrogel.

The samples were examined both under LV and with coating**.** The pore sizes, which very extensive, are shown with 150X (A) and 250X magnification (B). Since the amount of HA polymer is high, insufficient crosslinking formation is observed (C and D).

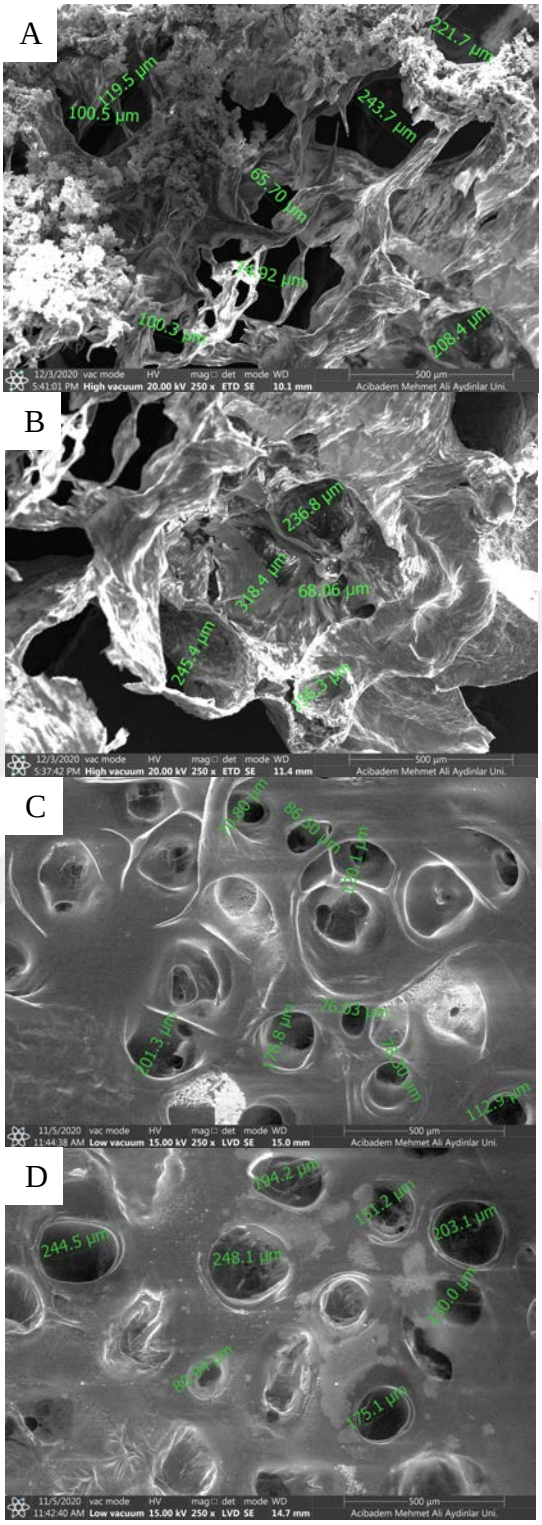

Figure 6.3. SEM images of ALG:HA:GEL 1:1:2 w/ $CaCl_2$ hydrogel.

The samples were examined both under LV and with coating**.** The pore sizes, which very extensive, are shown with 250X magnification (A, B, C and D).

In order to investigate the effects of hyaluronic acid and gelatin polymers in hydrogel form independently, ALG:HA (1:1) and ALG:GEL (1:1) hydrogels produced with the amounts indicated in Table 3 were crosslinked with $CaCl_2$ and two distinct control groups were obtained. SEM analysis of these control groups obtained from 1% alginate, 2% hyaluronic acid and 1% gelatin polymers and 50 mM $CaCl_2$ crosslinker was performed. When the images obtained from SEM analysis were examined, it is declared that the use of hyaluronic acid polymer increases the hydrogel stiffness and provides larger pores (Figure 6.4). On the other hand, it is declared that the use of gelatin polymers provides more crosslink bonding and thus a more stable hydrogel (Figure 6.5). However, according to the evaluation of swelling test results (4.2.1.), it was observed that both hydrogels were not enough reliable in PBS, except that ALG:HA model degraded more slowly. This outcome can be considered as an indication that the hyaluronic acid polymer enhances the hydrogel strength.

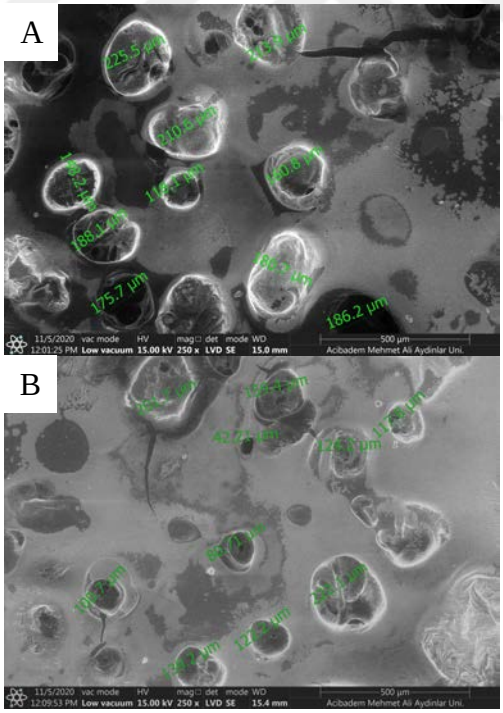

Figure 6.4. SEM images of ALG:HA 1:1 w/ $CaCl_2$ hydrogel.

The samples were examined under low vacuum. The pore sizes, which very extensive, are shown with 250X magnification (A and B).

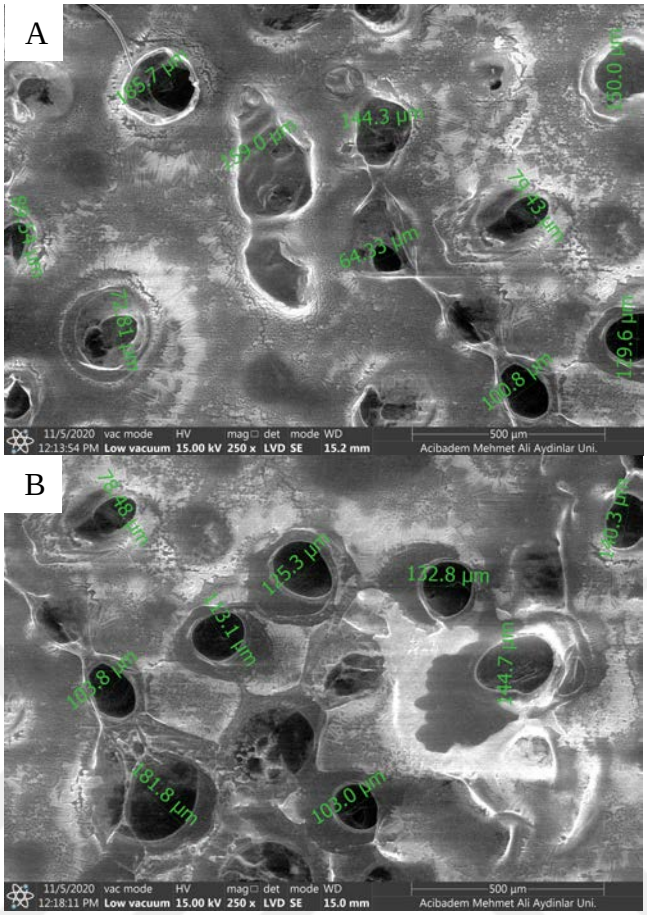

Figure 6.5. SEM images of ALG:GEL 1:1 w/ $CaCl_2$ hydrogel.

The samples were examined under low vacuum. The pore sizes, which very extensive, are shown with 250X magnification (A and B).

Subsequently, the morphology of the hydrogels achieved by applying various ratios of hyaluronic acid polymer concentration and $CaCl_2$ crosslinker molarity, keeping 4% alginate and 2% gelatin polymer concentrations constant, were analyzed with SEM. The samples were examined both under low and high vacuum.

Hydrogels were obtained as 75 mM $CaCl_2$ with 4% alginate, 2% hyaluronic acid, 2% gelatin and 50 mM $CaCl_2$ with 4% alginate, 4% hyaluronic acid, 2% gelatin. When these hydrogels were analyzed in SEM, it was observed that both formations had a

multi-pore structure and these pore sizes were wide (Figures 7.1 and 7.2). Both samples are considered to be compatible for *in vitro* studies.

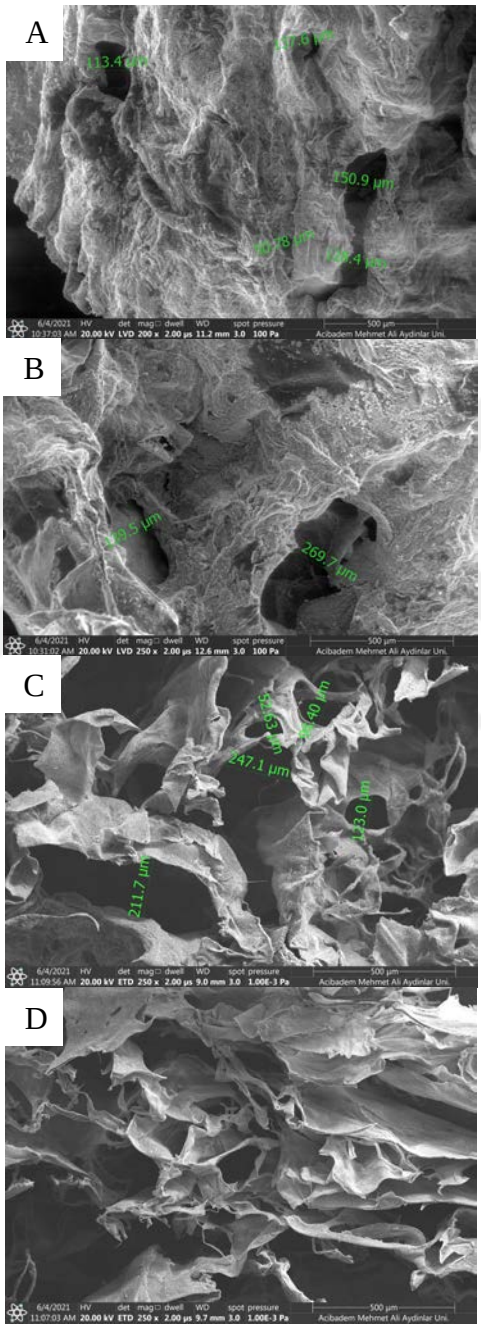

Figure 7.1. SEM images of 4%ALG-2%HA-2%GEL w/ 75 mM $CaCl_2$ hydrogel.

The samples were examined both under LV and with coating**.** The pore sizes, which very extensive, are shown with 200X (A), 250X magnification (B, C, and D).

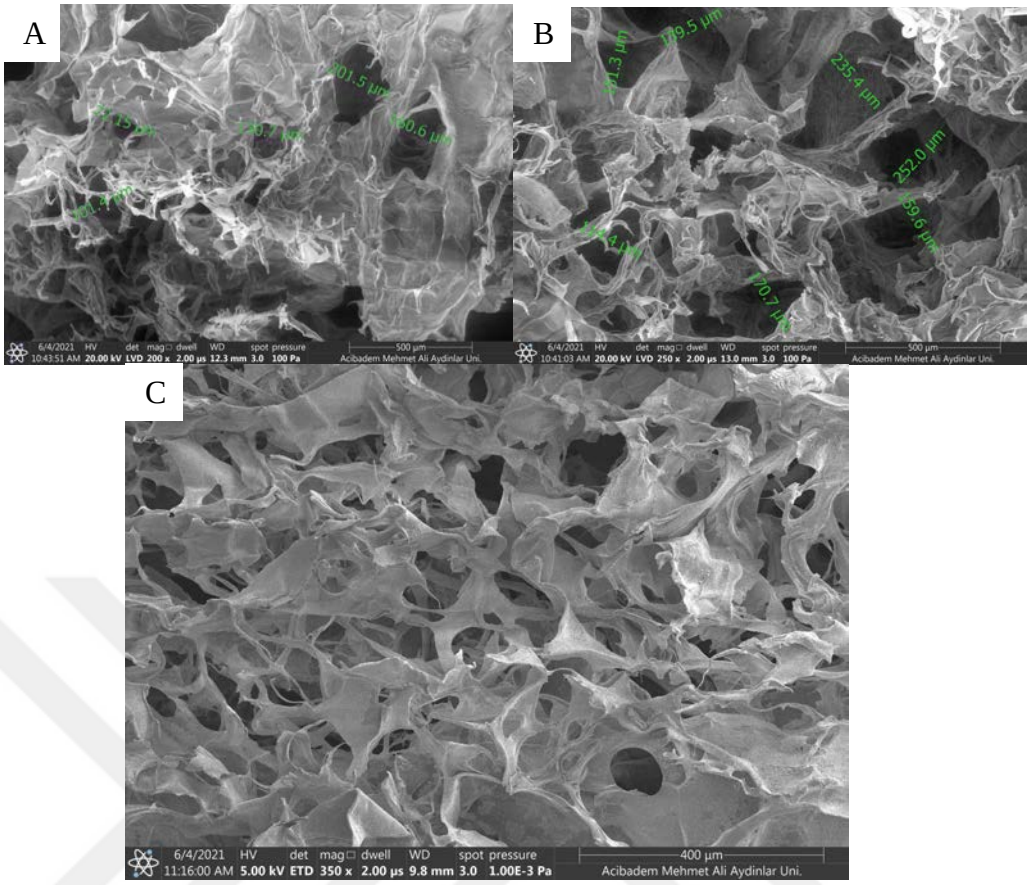

Figure 7.2. SEM images of 4%ALG-4%HA-2%GEL w/ 50 mM $CaCl_2$ hydrogel.

The samples were examined both under LV and with coating**.** The pore sizes, which very extensive, are shown with 200X (A), 250X magnification (B). The suitable porous structure is observed (C).

4.2.3. Rheological properties

Mechanical properties of hydrogels were analyzed by rheometric measurements with a UV-assisted rheometer (Kinexus, Malvern). In this measurement, continuously increasing and decreasing shear stress was applied to hydrogels at body temperature in a certain frequency range, to measure viscosity and deformation. In the charts shown below, G'' (as red curve) represents elastic modulus referring to solid-like state of the material and G' (as blue curve) represents viscous modulus referring to the liquid-phase of the material being analyzed. For hydrogels obtained by various weights,

percentages, and ratios of alginate, hyaluronic acid and gelatin, initial states represent gel-like property until cross point where G'' and G' overlap and as G'' and G' diverge hydrogel constructs starts to show deformation profile, which gets severe with increased stress, as it is expected. The graphs of the rheological properties of the hydrogels were examined according to this information are presented below.

η*: viscosity (as green curve), σ (°): phase angle, γ (%): shear strain

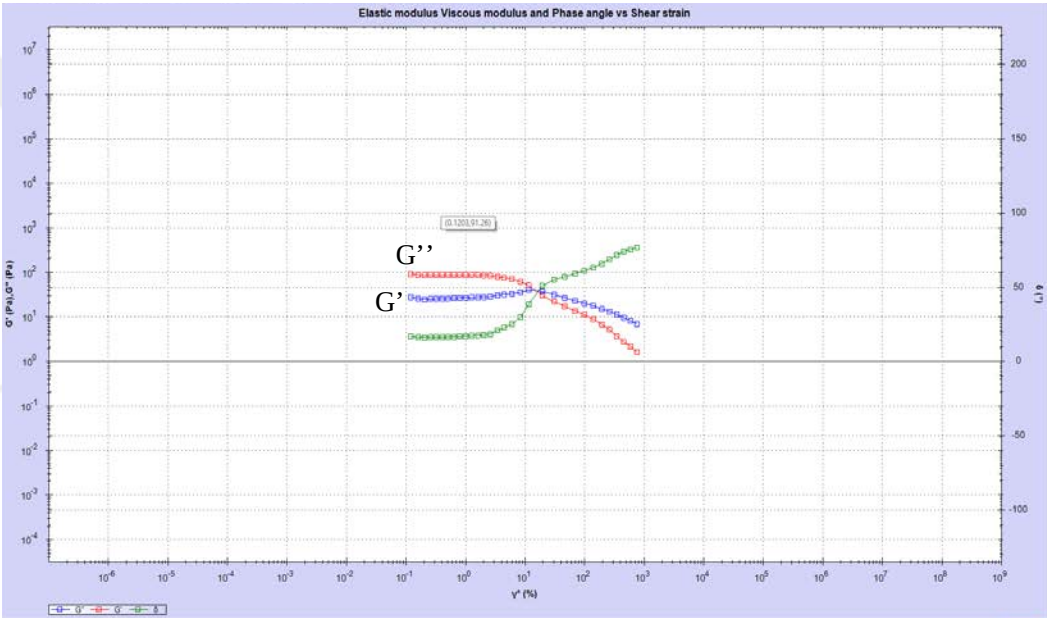

Figure 8.1. Rheology of HA:GEL 1:1 w/ GA hydrogel.

It is observed that deformation has occurred, indicating that it is gel-like structure.

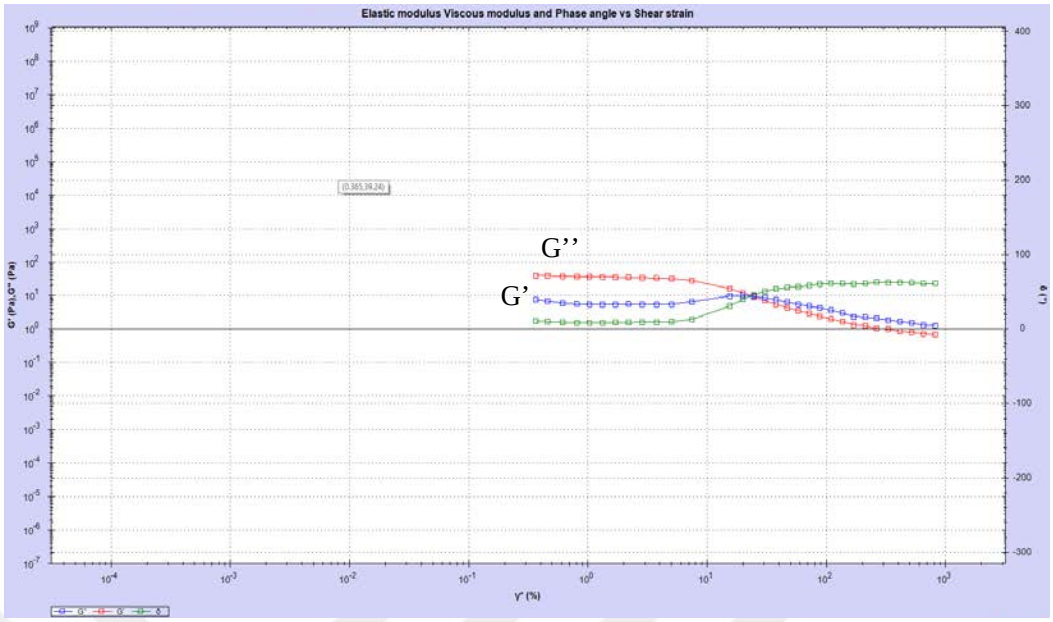

Figure 8.2. Rheology of HA:GEL 1:2 w/ GA hydrogel.

It is observed that deformation has occurred, indicating that it is gel-like structure.

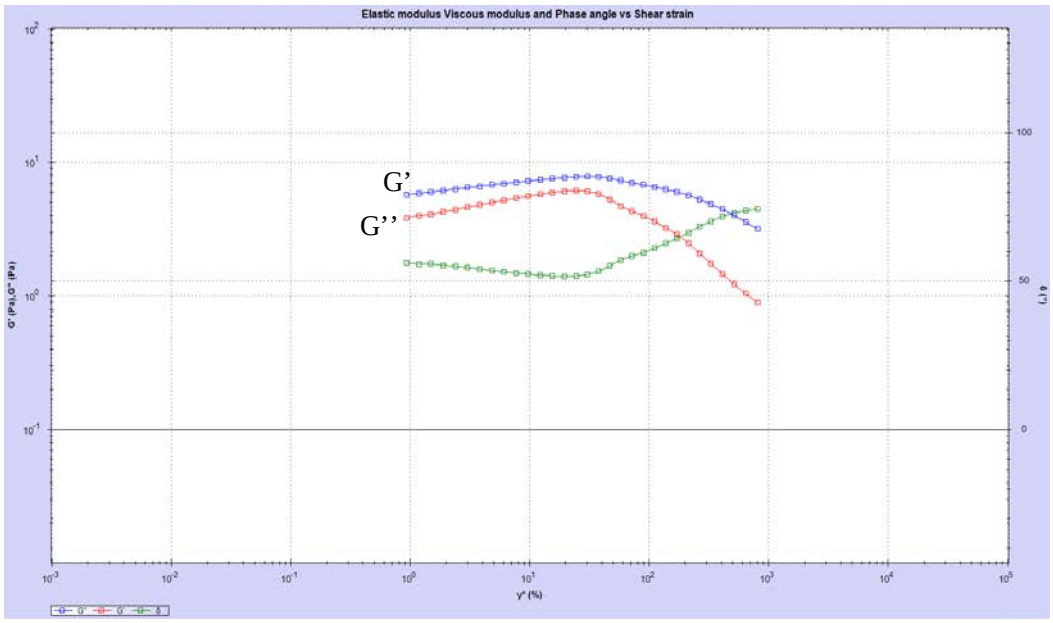

Figure 8.3. Rheology of HA:GEL 2:1 w/ GA hydrogel.

It is observed that deformation has not occurred, indicating that it is solid material.

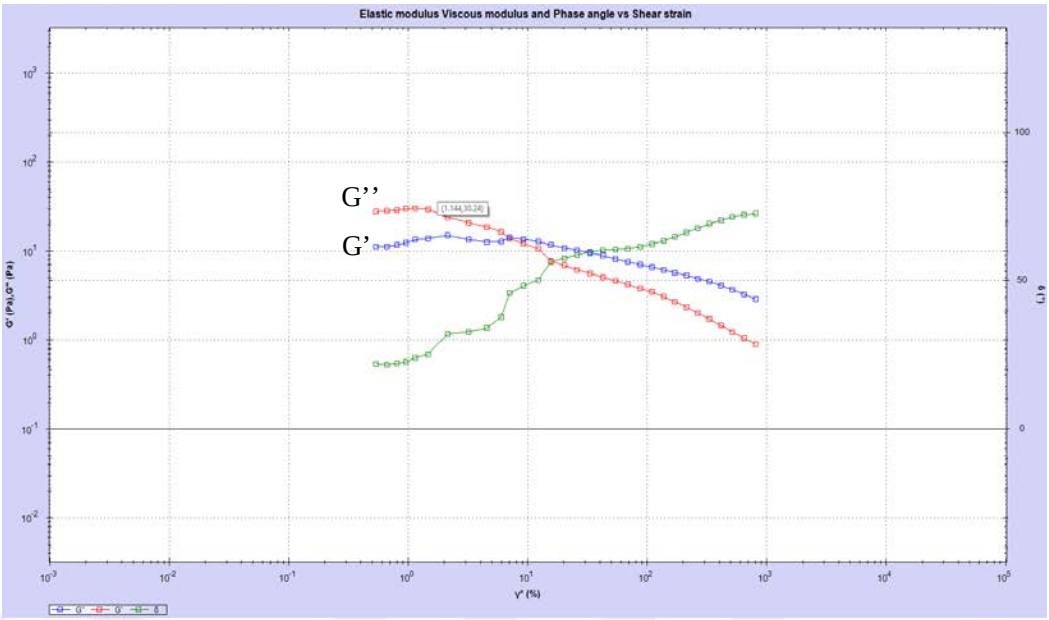

Figure 8.4. Rheology of HA:GEL 3:1 w/ GA hydrogel.

It is observed that deformation has occurred, indicating that it is gel-like structure.

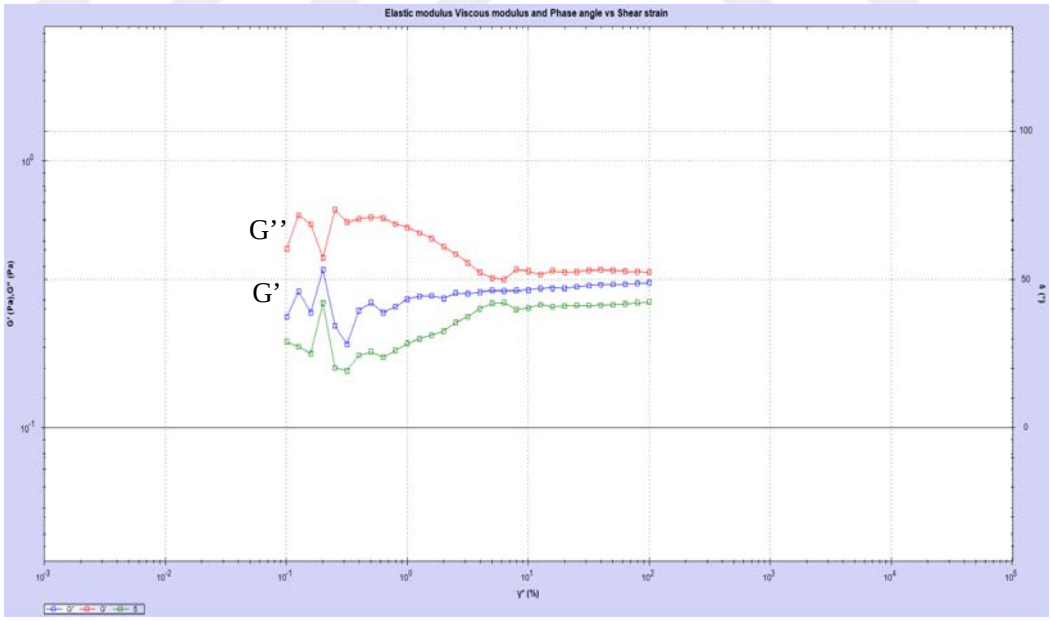

Figure 9.1. Rheology of ALG:HA:GEL 1:1:1 w/ $CaCl_2$ hydrogel.

It is observed that deformation has not occurred, indicating that it is solid material.

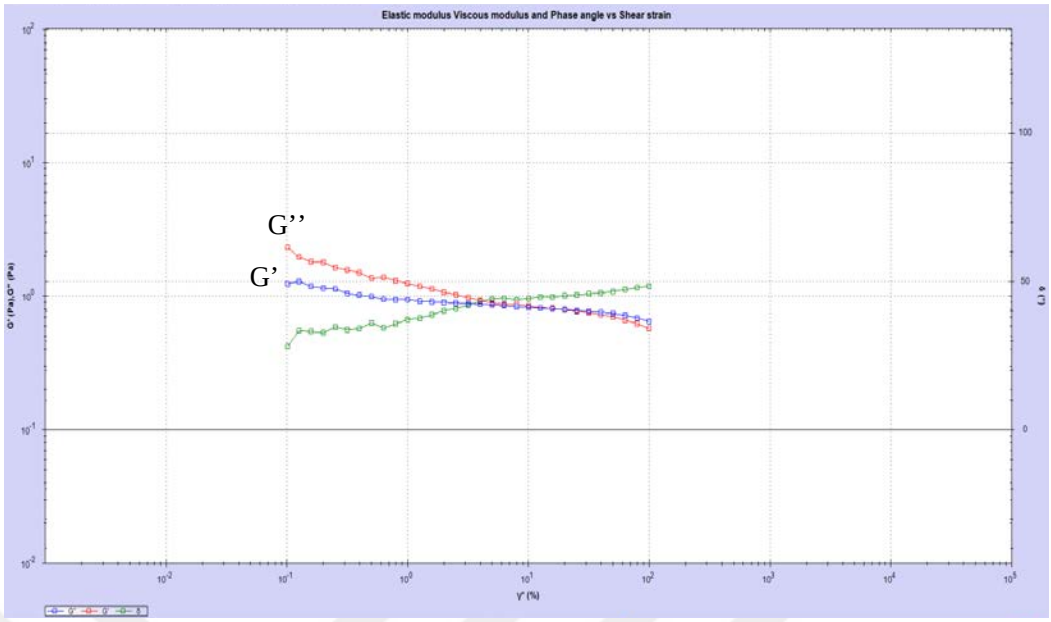

Figure 9.2. Rheology of ALG:HA:GEL 1:2:1 w/ $CaCl_2$ hydrogel.

It is observed that deformation has occurred, indicating that it is gel-like structure.

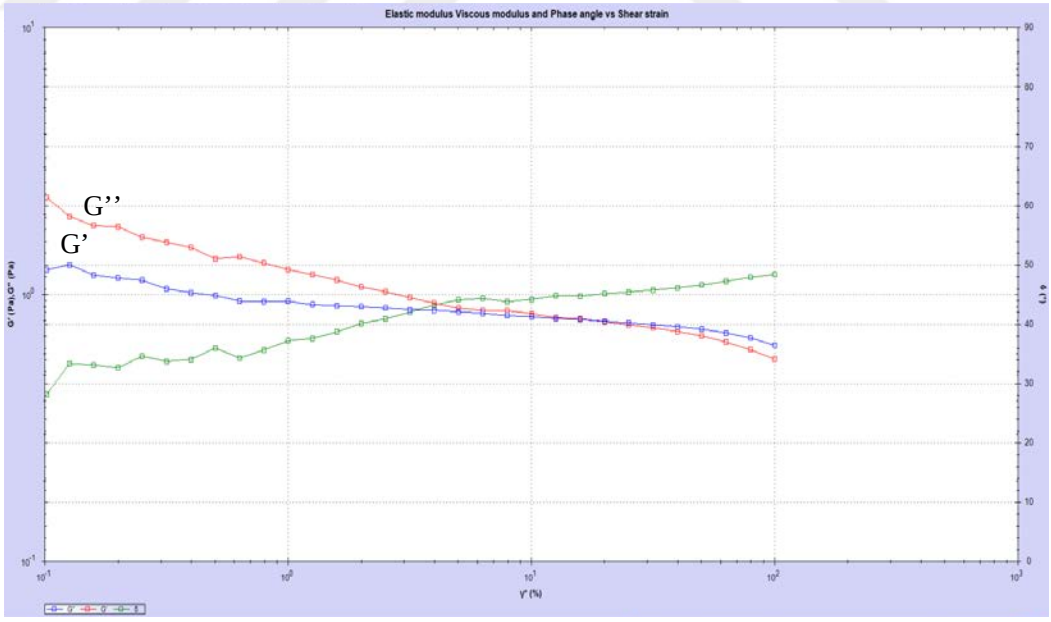

Figure 9.3. Rheology of ALG:HA:GEL 1:1:2 w/ $CaCl_2$ hydrogel.

It is observed that deformation has occurred, indicating that it is gel-like structure.

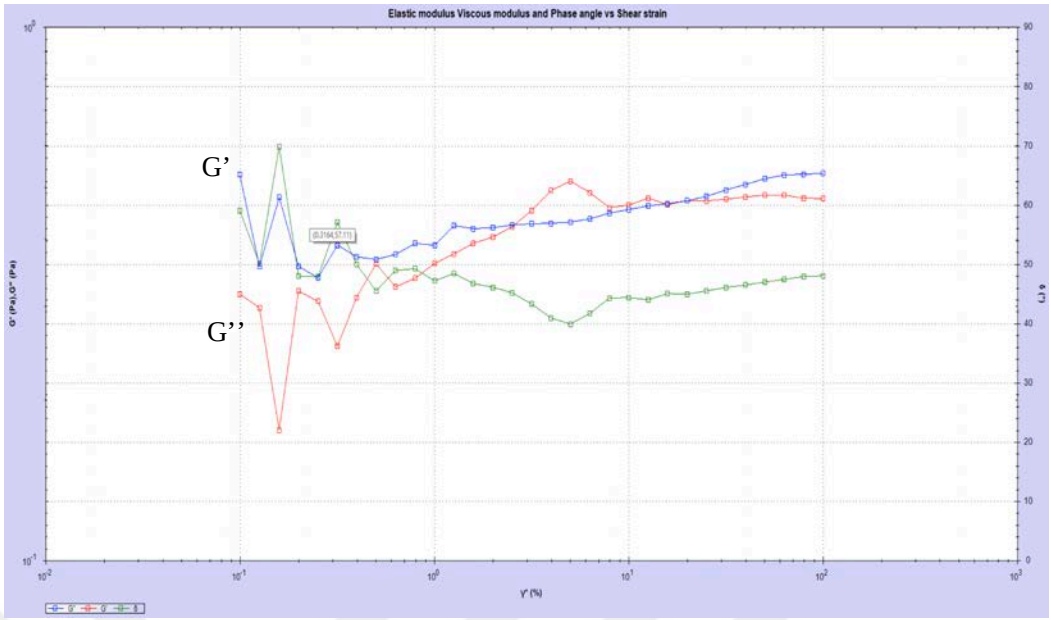

Figure 9.4. Rheology of ALG:HA 1:1 w/ $CaCl_2$ hydrogel.

It is observed that occurred a non-stable material.

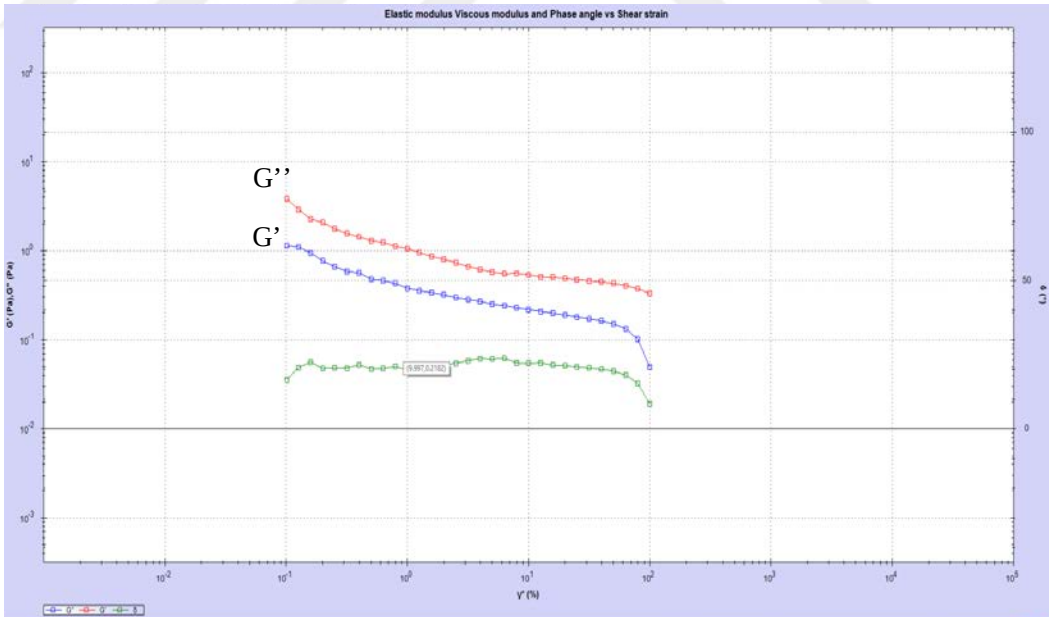

Figure 9.5. Rheology of ALG:GEL 1:1 w/ $CaCl_2$ hydrogel.

It is observed that deformation has not occurred, indicating that it is solid material.

Studies performed earlier, binary and triple combinations of alginate, hyaluronic acid and gelatin biopolymers were utilized with various ratios to achieve hydrogels with a high rate of conversion. According to obtained rheometric results, HA:GEL 2:1 (Figure 8.3), ALG:HA:GEL 1:1:1 (Figure 9.1), and ALG:GEL 1:1 (Figure 9.5) hydrogels did not present any deformation at the working interval frequency during the measurements. All other hydrogels prepared with different ratios of these polymers demonstrated a deforming profile upon an increase in the stress and frequency during the measurement. Only ALG:HA 1:1 (Figure 9.4) hydrogel showed a very non-stable behavior.

The rheometric results of the two hydrogels achieved in the last experimental group were once compared, it is observed that hydrogel scaffold with less (2%) content of hyaluronic acid (Figure 10.1) begins to deform earlier at lower frequencies compared to the one with more (4%) hyaluronic acid content (Figure 10.2). This result is compatible with literature results, which is attributed to the stiff nature of hyaluronic acid hydrogel due to string polar-polar interactions of hyaluronic acid chains with each other at aqueous media.

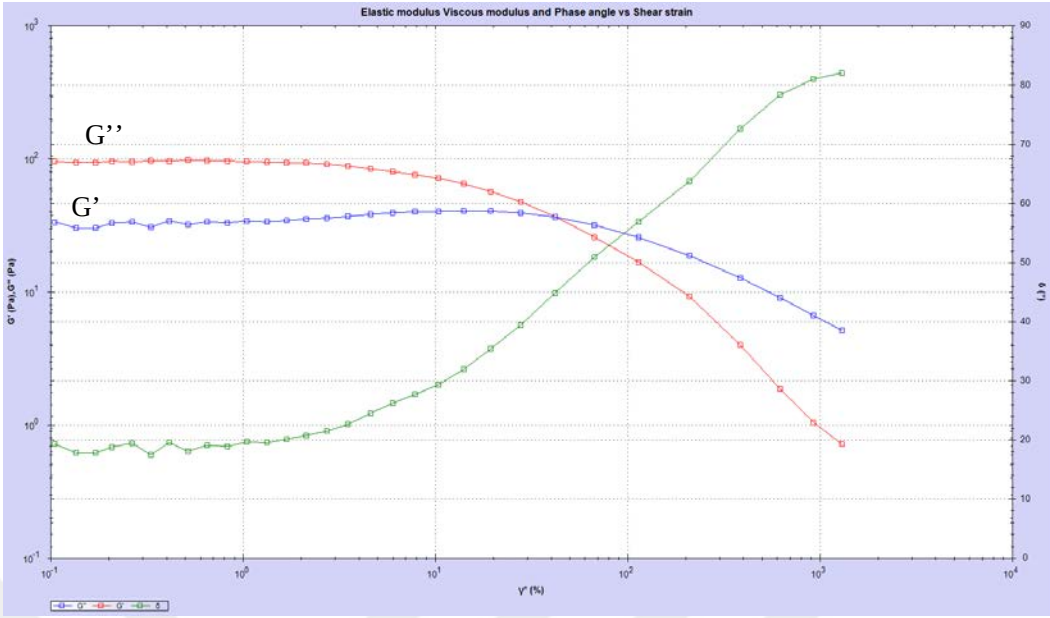

Figure 10.1. Rheology of 4%ALG-2%HA-2%GEL w/ 75 mM $CaCl_2$ hydrogel.

It is observed that deformation has occurred, indicating that it is gel-like structure.

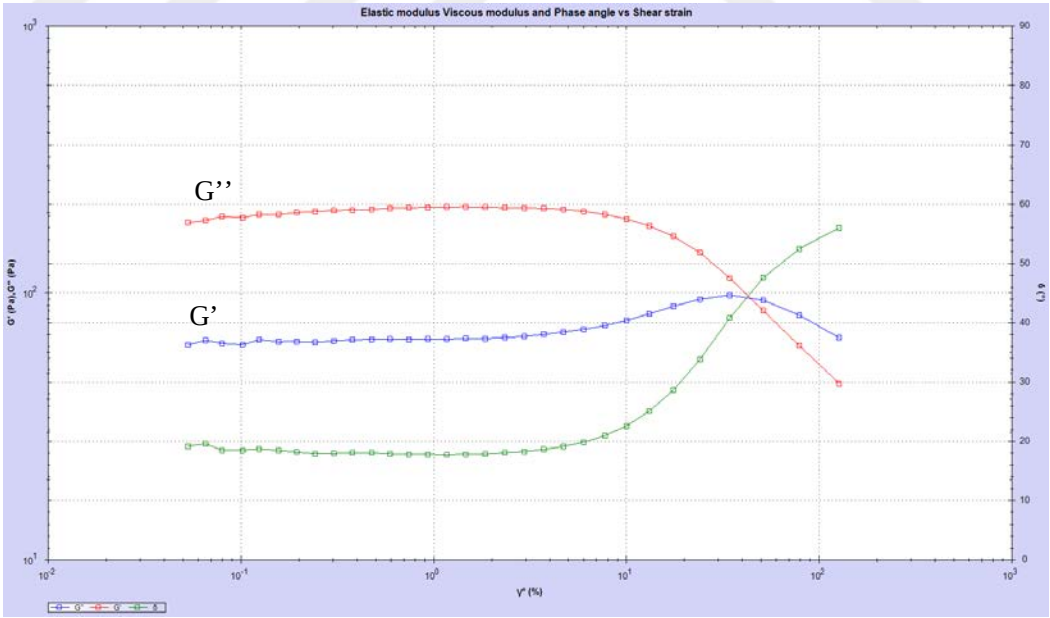

Figure 10.2. Rheology of 4%ALG-4%HA-2%GEL w/ 50 mM $CaCl_2$ hydrogel.

It is observed that deformation has occurred, indicating that it is gel-like structure.

4.2.4. FT-IR spectroscopy analysis

Prepared hydrogels with the combination of three different natural biopolymers, alginate, hyaluronic acid and gelatin were characterized for their chemical compositions and functional groups via FT-IR Spectroscopy technique. Analyzes were performed with Nicolet™ iS™ 10 FTIR Spectrometer (Thermo Scientific™). Comparative results are presented in Figure 11.1 and Figure 11.2, with the curves overlapping.

Hydrogels differing in their hyaluronic acid content were investigated (Figure 11.1). Hydrogels were crosslinked physically with the different concentration of $CaCl_2$ solution. Labile proton bonds in carboxylic acid groups (-COO-H), hydroxyl groups (O-H) and amine/amide bonds (N-H) can be observed as a broad peak around 3300 cm^{-1}, as expected. Aliphatic (–C-H) peaks can be observed at 3300 cm^{-1}. The carbonyl peaks coming from amide carbonyl groups in gelatin structure, carboxylic acid group of hyaluronic acid and alginate can be seen as an overlapped multiple peak at around 1600 cm^{-1}. Specifically, the peaks between 1060-1020 cm^{-1} frequencies represent C-O bonds, which is found abundantly in hyaluronic acid structure. Therefore, this result proves the hyaluronic acid content comparably for the hydrogel containing more hyaluronic acid in its structure (ALG:HA:GEL vol. ratios with 4:4:2).

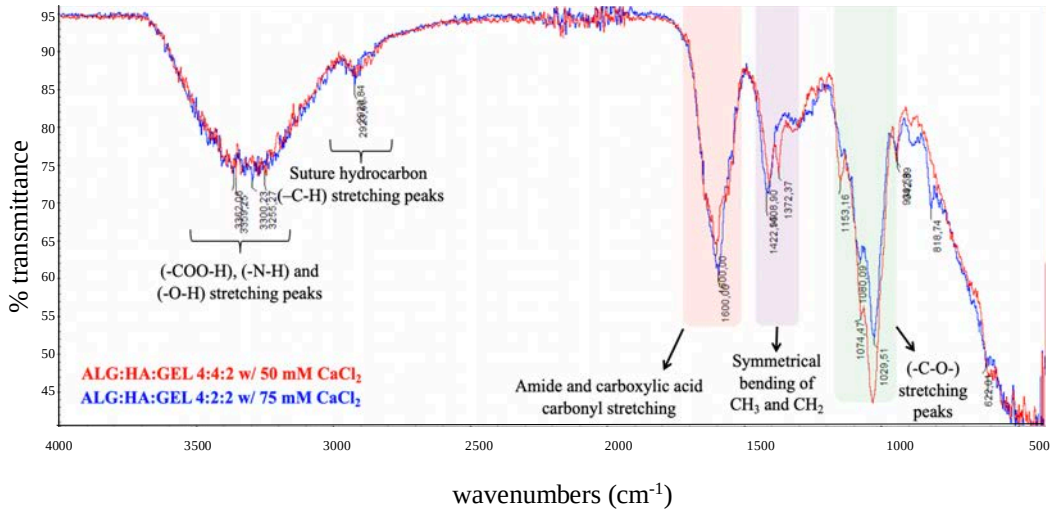

Figure 11.1. FT-IR spectra of hydrogels differing in their HA content

In order to control, hydrogels with increasing the amount of non-crosslinked polymer types were prepared and compared with the one involving same polymer percent (ALG:HA:GEL vol. ratios with 1:1:1). FT-IR spectra of prepared control hydrogels can be seen in Figure 11.2. As compared to the hydrogel obtained from ALG:HA:GEL with 1:1:2 vol. ratio, doubling of gelatin content leads to an increase in intensity of the peak observed at around 1640 cm^{-1}, which belongs to amide carbonyl groups coming from gelatin structure. On the other hand, doubling of hyaluronic acid content in the hydrogel scaffold seems to contribute to a remarkable increase in the intensity of C-O peaks observed at 1226 cm^{-1}, which is expected because of the presence of abundant C-O bonds based functional groups in hyaluronic acid chemical structure.

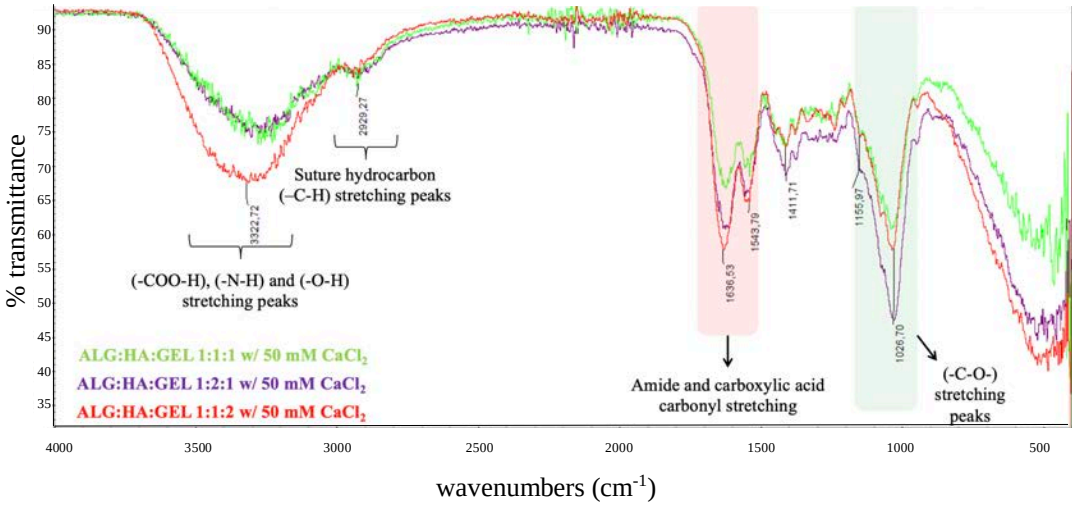

Figure 11.2. FT-IR spectra of hydrogels ALG:HA:GEL with different ratios

4.2.5. Histology

Fixation was applied to the samples obtained 48-72 hours after hydrogels prepared in 24-well-plate to be examined in SEM. Samples were fixed overnight in 2.5% glutaraldehyde fixative with 0.1 M PBS buffer (pH 7.2). After washing with tampon, postfixation was done with 1% osmium tetroxide for an hour. The samples were dehydrated by passing through a series of ascending alcohols (70%, 90%, 96%, 100%). Following the 3/1, 1/1 and 3/3 alcohol/amylacetate series after dehydration, the samples placed in pure amylacetate were covered with gold after drying in the critical point dryer and examined under a scanning electron microscope.

Since the hydrogels were partially degraded, very limited images could be obtained from the samples analyzed in SEM. However, formations similar to NK-92 cells were observed (Figure 12.1). These formations, are seen to be similar by comparison with the literature (Figure 12.2), and is considered to be NK-92 cell (69). Furthermore, the dimensions of the diameter-measured formations have been

confirmed by determining that they are between 12-18 µm in the literature research (70).

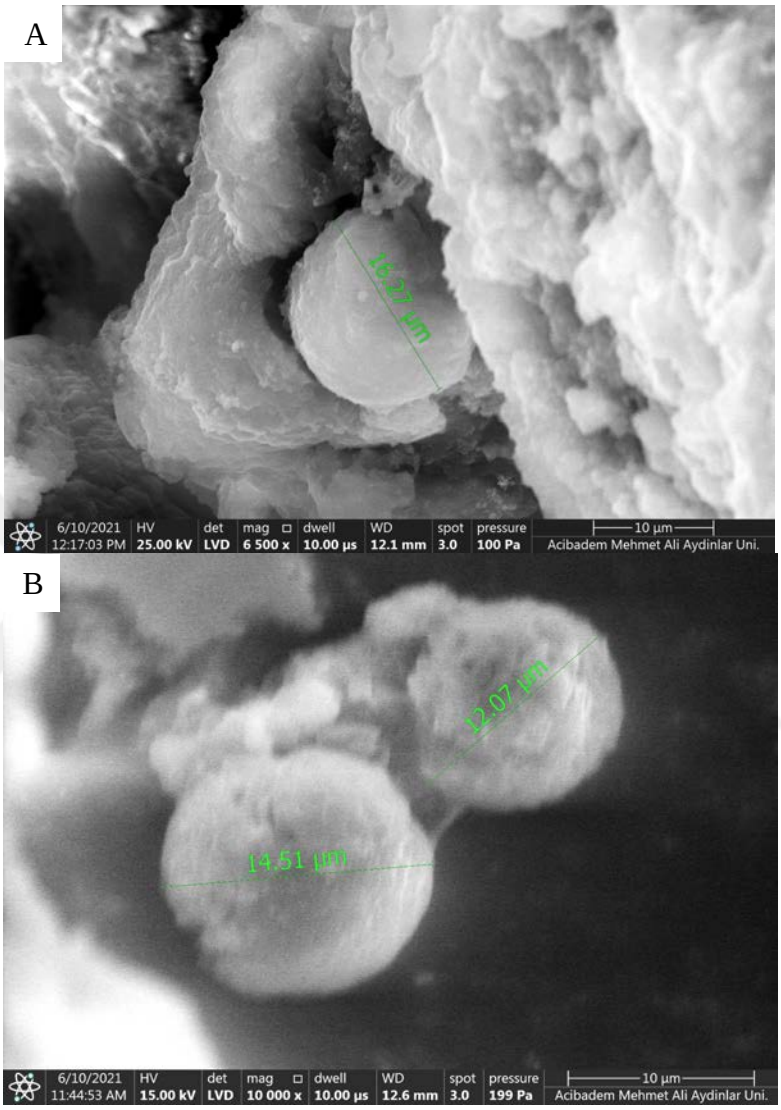

Figure 12.1. SEM analyzing of cell-laden hydrogel.

The cell-like structures indicated by the diameters are considered to be NK-92 cells compared to the representative image below.

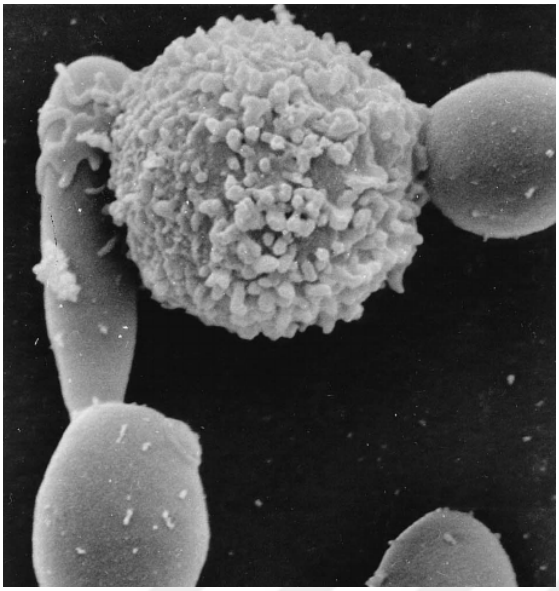

Figure 12.2. Representative sample of NK-92 cell in SEM analyzing (Arancia et al., 1995).

The data obtained with this histological analysis were evaluated, as a consequence it was observed that the cells could remain viable and adhere to the hydrogel architecture in the 3D scaffold designs provided by seeding NK-92 cells into these optimized hydrogels, which is a corporation not found in the literature before.

5. DISCUSSION AND CONCLUSION

The choice of biomaterials has a critical role in the improvement of therapeutic implants and tissue-engineered products. Essentially, a safe and stable material should be inert, thus that the artificial material placed in the patient's body should not lead to a cellular response. The inertness of biomaterials is a highly effective consideration for the achievement of many applications such as providing support in the accomplishment of cellular activities for the growth of new tissue, prevention of cellular activity in situations where tissue growth is undesirable in surgical interventions, maintaining desired cellular interactions with directed tissue response, enhancing of cell attachment and subsequent cellular activation, inhibition of cellular attachment and/or activation, avoidance of any biological response (2).

3D polymer hydrogel scaffolds, which is biodegradable, biocompatible and have low immunogenicity properties, have a hydrophilic pore structure that allows massive water absorption, and with the high amount of water in their composition support integrity, solubility and diffusion of substances. These properties are effective factors in delivering therapeutic agents. Its soft structure, elasticity and similarity to living tissues make hydrogels an excellent tool in tissue engineering and regenerative medicine applications, as well as in stem cell cultures and cell implantations. The 3D porous scaffolds mimic the ECM *in vitro* and this ensures cell proliferation and differentiation with the artificial ECM construct. The hydrogels obtained in this thesis were observed in accordance with the literature and, as indicated in 4.2.2., in SEM analysis, it was considered that the pore sizes were suitable for the attachment of cells and the transportation of substances. Cell viability and mechanical stability of the hydrogel are enhanced when appropriate crosslinkers and ratios are applied in the design of hydrogel scaffolds. Furthermore, it is an excellent dressing material due to its non-adhesive nature, malleability and resemblance to living tissue. It provides a moist environment to stimulte cell proliferation and accelerate the wound healing

process. In particular, for burns and painful wounds, they sooth the wound tissue and reduce pain (49).

Hydrogels, one of many *in vitro* and *in vivo* applications developed in tissue engineering, are also applied for many skin injuries. They may consist of a polymer or polymer composites, either naturally-derived or synthetically. When more than one polymer is used collectively, it is aimed to achieve a stronger material by various physical or chemical methods. Therefore, it is crucial to choose more compatible biomaterials. The biocompatible and non-toxic nature of biomaterials, particularly in hydrogel designs to be combined with cell culture, is a highly serious consideration *in vitro* studies. Choosing polymers that any cell group is adapted to from natural tissues can increase the success rate in hydrogel design. Considering these features, many studies have been carried out. Singh et al. have designed gelatin, alginate and hyaluronic acid-based hydrogels prepared at various concentrations in their study. Hyaluronic acid was fixed at 10% concentration, while alginate and gelatin were applied at different concentrations. They reported that a mechanically stable hydrogel was achieved when the alginate concentration was 2% or 2.25% and the gelatin concentration was 2.25%. The optimal hydrogel matrix they obtained has been shown to promote the scaffold stability and cellular activities. In this study, they showed that MSCs provided satisfying results in proliferation, differentiation and mineral deposition (5). Sakai et al. have investigated the viscosity of gelatin-hyaluronic acid-based hydrogel scaffolds prepared at various concentrations, the gelling behavior of polymer solutions as well as the printability properties. The mechanical and molecular permeability properties of resultant hydrogels and their effects on hASCs (human adipose-derived stem cells) were examined. It has been shown that increasing the polymer content does not consistently lead to an increase in viscosity of the solution (57). Ratanavaraporn et al. have produced hydrogel scaffold using ionic crosslinker ($CaCl_2$) with a mixture of hyaluronic acid and alginate with different weights but at the same concentration (1%). As a result of this study, it was observed that the use of hyaluronic acid at a high rate increased water absorption, but accelerated degradation (58).

Alginate, hyaluronic acid and gelatin, the polymers were utilized in this thesis, demonstrate good mechanical strength as non-toxic biomaterials and have high biocompatibility. While the alginate polymer can only form a gel by crosslinking divalent cations such as Ca^{+2} and Ba^{+2}, hyaluronic acid requires chemical modification. Therefore, at the beginning of the studies, it was aimed to eliminate of the chemical crosslinker by adding alginate polymer to the hyaluronic acid-gelatin composite crosslinked with glutaraldehyde. The main reason for applying $CaCl_2$ instead of glutaraldehyde to the developed hydrogels is that it provides rapid gelation and is more relevant for cell encapsulation as it is a physical crosslinking agent. Since glutaraldehyde provides chemical crosslinking, it is more suitable for systems where cells are transplanted into pre-prepared hydrogels. However, it is not sufficient as it is a reactive agent *in situ* gelling by combining polymers and cells collectively, as practised in this thesis. Gelatin polymer, which has cell-interactive functional groups, develops cell adhesion properties in the hyaluronic acid hydrogel scaffold. The combination of alginate-gelatin supplemented with $CaCl_2$ also produces a suitable composite for cell adhesion and proliferation properties. All these well-ordered effects are the considerations of these polymers were chosen for cell-laden hydrogel design. The use of high hyaluronic acid content resulted in larger pore sizes of the hydrogel scaffold. Furthermore, the combination of hyaluronic acid-gelatin increased the compressive strength of the hydrogel scaffold. Corresponding to the swelling test results, it was observed that the amount of water taken into the hydrogel increased with a high concentration of hyaluronic acid. Besides, higher concentration resulted in larger pores, as examined in SEM images. This porous scaffold structure obtained allows the cells to attach to the hydrogel, their proliferation, and nutrient and metabolite exchanges.

The hydrogels studied in this thesis were firstly hyaluronic acid-gelatin based and crosslinked with glutaraldehyde, and their mechanical, chemical and morphological properties were examined. Along with the data collected from the results, this hydrogel was crosslinked with glutaraldehyde by adding alginate polymer, and with $CaCl_2$ to elimination of glutaraldehyde. $CaCl_2$, which has been applied to the hydrogels,

provides a physical (ionic) crosslinking. In order to investigate the effects of hyaluronic acid and gelatin polymers separately, control groups in the form of alginate-hyaluronic acid, alginate-gelatin were prepared, and it was observed that the use of hyaluronic acid was significantly effective in gel formation. At the last stage, it was determined to crosslink the alginate-hyaluronic acid-gelatin-based hydrogels with $CaCl_2$, and this point the polymer concentrations were increased to make the hydrogel scaffold structure more stable. Hydrogels were produced by preparing hyaluronic acid in two different (2% and 4%) concentrations by keeping alginate (4%) and gelatin (2%) concentrations constant. *In vitro* studies were performed on these two hydrogels, which were observed to have better mechanical, chemical and morphological properties. In these *in vitro* studies, unlike the hydrogels obtained with many cell types, the NK-92 cell line used makes a difference compared to the literature. In recent years, specifically after the development of CAR-T cell therapies, researches on NK-92 have been on the rise. NK-92, which has been studied particularly in melanoma cancer types, does not lead to cellular response since it has no immunological markers, thus it can be used as an allogenic source.

NK-92 cell culture was optimized with three different media and two candidates, TexMACS and X-Vivo 15, were found to be quite successful. The use of these media was found to be compatible with many studies in the literature. The disadvantage of designing a model by seeding NK-92 cells into hydrogel scaffolds is the consideration of the cell attachment by the reason of it is a suspended cell. In order to detect possible shortcomings, adherent HEK293T cells were also seeded into the two hydrogels obtained as a result of optimization, in addition to the suspended NK-92 cells. As a result of the analysis, the pore size increased with the hyaluronic acid concentration, and its mechanical durability became better. However, the degradation was occurred rapidly which is not intend. The concentrations of the same polymers and the molarity of the $CaCl_2$ crosslinker can be increased to improve the degradation properties. The results obtained showed that NK-92 cells were able to survive in the hydrogel form, as indicated in the SEM analysis. However, no data on the presence of HEK293T cells in the hydrogels were obtained.

The aim of these studies is to contribute to cell-laden 3D hydrogel scaffold optimizations found in the literature. In the hydrogels designed with references from various studies, the types of the polymers and their concentrations were determined with specific parameters and the optimization trials of more than one preparation were performed. Along with the unsuccessful hydrogels, the designs that were found to be partially successful as a result of the study were achieved. In order to open the path for prospective studies and present a different perspective, the NK-92 cells, which is a cell line applied as only cellular therapy particularly in cancer patients, has been seeded in the hydrogels that can remain stable even for a relatively short time. In future strategies, it is considered that these hydrogels can be enhanced with different modifications. Besides, injectable forms of these polymer-cell mixtures can also be obtained in prospective studies. Furthermore, it was assumed that the cellularly applied NK-92 therapies could be applied as a treatment in a tissue engineered tool. Although the proliferation of NK-92 cells could not be detected completely with the results obtained, their ability to preserve their viability in the produced hydrogels enabled this thesis to reach the purpose for which it was developed.

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7. CURRICULUM VITAE