MARMARA UNIVERSITY

INSTITUTE FOR GRADUATE STUDIES

3D PRINTING OF SOFT TISSUE ENGINEERED CONSTRUCTS FOR SKIN RECONSTRUCTION

HATİCE MERVE CAN

MASTER THESIS

Department of Bioengineering

Thesis Supervisor

Prof. Dr. Faik Nüzhet OKTAR

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Prof. Dr. Mehmet Eroğlu

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Hatice Merve CAN, a Master of Science student of Marmara University Institute for Graduate Studies in Pure and Applied Sciences, defended her thesis entitled "**3D Printing of Soft Tissue Engineered Constructs for Skin Reconstruction**", on December 23, 2019 and has been found to be satisfactory by the jury members.

Jury Members

Prof. Dr. Faik Nüzhet OKTAR (Advisor)
Marmara University .....

Assoc. Prof. Oğuzhan GÜNDÜZ (Jury Member)
Marmara Üniversitesi .....

Assoc. Prof. Cem Bülent ÜSTÜNDAĞ (Jury Member)
Yıldız Teknik Üniversitesi .....

APPROVAL

Marmara University Institute for Graduate Studies in Pure and Applied Sciences Executive Committee approves that Hatice Merve CAN be granted the degree of Master of Science in Department of Bioengineering, Bioengineering Program on 02.01.2020
(Resolution no: 2020/01-02).

Director of the Institute
Prof. Dr. Bülent EKİCİ

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"Wishing to see and live the days when science is used for the benefit of humanity and justice and merit prevail both in my country and in the world."

Hatice Merve CAN

TABLE OF CONTENTS

ÖZET

DERİ YENİLENMESİ İÇİN 3 BOYUTLU YAZICI İLE YUMUŞAK DOKU MÜHENDİSLİĞİ YAPILARININ ÜRETİMİ

Jelatin – metakrilamid hidrojellerin doğal polimerler ile 3 boyutlu yazıcılar kullanılarak üretilen yumuşak doku uygulamaları, deri rejenerasyonu açısından büyük potansiyel taşımaktadır.

Bu tez çalışmasında, doku yapılarını taklit eden yapay bir yapı oluşturmak için jelatin ile metakrilik anhidrit tepkimesinden gelatin-metakrilamid sentezi ile bir başka doğal polimer olan hyaluronik asit katılarak oluşturulan 3 boyutlu mesh yapılar oluşturulması ve bu mesh yapıların vücutla uyumluluğunu araştırılması için hücre canlılık testinin gerçekleştirilmesi amaçlanmıştır. Bu bağlamda, jelatin-metakrilat sentezlenmiş ve çapraz bağlı hidrojel oluşturmak üzere foto başlatıcı ilave edilmiş, ardından 3-boyutlu jelatin-metakrilat hiyalüronik asit karışımları ile birlikte ve üç boyutlu jelatin-metakrilat oluşturulmuş ve ultraviyole lamba altında çapraz bağ oluşumu sağlanmıştır. Jelatin-metakrilat ve hyaluronik asit-metakrilat yoğunluğu, viskozitesi/reolojik karakterizasyonu, karakterizasyon testleri açısından baskı öncesi hidrojellerin yüzey gerilimi testleri gerçekleşitirilmiştir. Daha sonra optik mikroskop, Fourier Transform Infrared Spektroskopi analizleri ve taramalı elektron mikroskopisi ile morfolojik, boyutsal ve kimyasal analizler yapılmıştır. Bu testlerin ardından yumuşak doku yapılarının mekanik ve termal analizleri yapılmıştır. Ek olarak, 3 boyutlu yapıların hücre canlılığına etkisi için fibroblast hücreleri üzerinde hücre kültürü testleri ile gerçekleştirilmiştir.

Sonuç olarak, jelatin-metakrilat hidrojel ve kompleks hyaluronik asit ile oluşturulan 3-boyutlu yapıların karakteristik özellikleri belirlendi ve hücre canlılığı üzerinde sitotoksik özellik ve etki göstermediği tespit edilmiştir. Bu nedenle, jelatin-metakrilamit hidrojel ile hyaluronik asit metakrilamit katkılı gelatin metakrilamit 3 boyutlu yapılarının, yumuşak doku mühendisliği uygulamaları için büyük bir potansiyele sahip olduğu tespit edilmiştir.

ABSTRACT

3D PRINTING OF SOFT TISSUE ENGINEERED CONSTRUCTS FOR SKIN RECONSTRUCTION

Soft tissue applications of gelatin - methacrylamide hydrogels produced by using natural polymers and 3D printers have great potential for skin regeneration.

In this thesis, it is aimed to print 3 dimensional mesh structures by adding gelatin-methacrylamide, that are reacted gelatin and methacrylic anhydride, and hyaluronic acid which is another natural polymer to form an artificial structure that mimics tissue structures and to investigate the cell viability test for the compatibility of these mesh structures with the body. In this context, gelatin-methacrylate was synthesized and added to the photoinitiator to form cross-linked hydrogel, followed by 3-dimensional gelatin-methacrylate with mixtures of hyaluronic acid, and mesh structures were formed, and exposure under ultra-violet lamp for cross-link. Density, viscosity/rheological characterization of gelatin-methacrylate and hyaluronic acid-methacrylate, surface tension of hydrogels before printing in terms of characterization tests were performed. After then, morphological, dimensional and chemical analysis were done with optical microscope, Fourier Transform Infrared Spectroscopy analyzes and scanning electron microscopy. Following these tests, mechanical and thermal analysis of soft tissue structures were done. In addition, the skin cells were cultured in 3-dimensional scaffolds with different structures and the cellular properties of the 3-dimensional structure were investigated by cell culture tests.

As a result, the characteristic properties of 3-Dimensional structures formed with gelatin-methacrylate hydrogel and complexed hyaluronic acid were determined and showed no cytotoxic properties and effects on cell viability. Therefore, gelatin-methacrylate hydrogel, and gelatin-methacrylate with hyaluronic acid-methacrylate hydrogel have a great potentials for soft tissue engineering applications.

SYMBOLS

% **:** percent

& **:** and or with

~ **:** approximate

< : less than

> : greater than

µL : microliter

µm : micrometer

cm : centimeter

w/v **:** weight by volume

v/v **:** volume by volume

° C : Celcius

h : hour

M **:** molar

ml : milliliter

g : gram

mN/m **:** milli newton per meter

rpm : revolutions per minute

mPa s : millipascal second

M_w/M_n : Molecular weight

X : Swelling ratio for 3D structures

W_1 : Wet weight for 3D structures in swelling test

$W_{1,dry}$ **:** Dry mass of 3D structures in swelling test

Y **:** Degradation ratio

$\mathbf{W_2}$: Wet weight for 3D structures in degredation test

$\mathbf{W_{2,dry}}$ **:** Dry mass of 3D structures in degredation test

ABBREVIATIONS

3D **:** Three dimensional

UV **:** UltraViolet

GelMA **:** Gelatin – methacrylate

HA **:** Hyaluronic acide

HAMA **:** Hyaluronic acid – methacrylate

DSC **:** Differential Scanning Calorimetry

SEM **:** Scanning electron microscopy

FTIR **:** Fourier Transform Infrared Spectroscopy

ECM **:** Extracellular matrix

LAP : Lithium phenyl-2,4,6-trimethylbenzoylphosphinate

PBS : Phosphate-buffered saline

DMEM : Dulbecco's Modified Eagle Medium

CO_2 : Carbon dioxide

MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

RPM : Revolutions per Minute

DMSO : Dimethylsulfoxide

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1. Tissue Engineering Applications

Tissue engineering is an interdisciplinary field that implements the principles of engineering and life sciences to the improvement of biological materials/ products that reinstate, protect or develop tissue functionality or a whole organ, as seen today. This uses the scaffold matrices to fill the tissue cavity, ensure structural support, and present growth factors and / or cells capable of forming tissue in the body upon transplantation. The tissue engineering/ regenerative medicine strategies are required to cellular interaction and entegration by including appropriate physical and cellular signalizations. So that, it has crucial importance to include modifying factors such as the active proteins and DNA as biological. Nowadays, more simple procedures are more successful and it means that usage of skin cell layers are more useful instead of using damaged cartilage. In addition, bigger and more complex skin reconstructions, especially bladder, it was performed hopefully for more and more complex tissue engineering applications for future. Even though the basic functional tissue engineering strategies are key, there is still important area for immune modulations, next improvements of cell sources, cell supports induvidually, vacularization and abilities of computer and mathematical modelling [1].

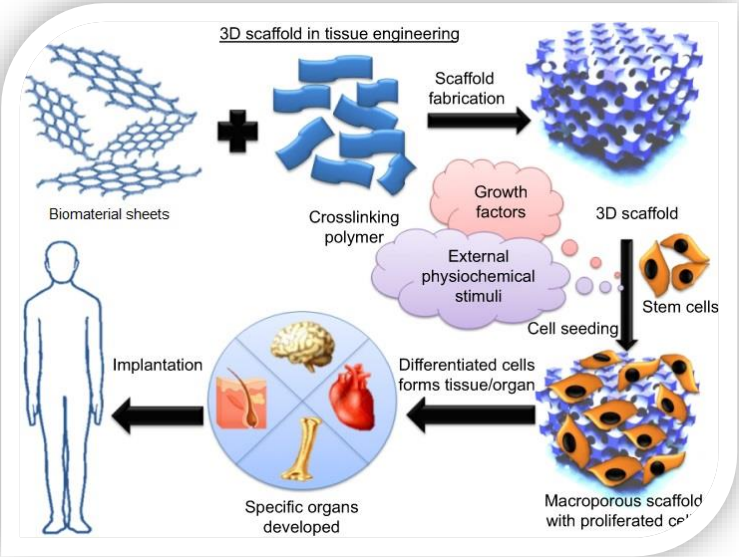


Figure 1.1. 3-dimensonal scaffold in tissue engineering

The damage and degeneration of tissues are generally observed due to trauma, disease, and injury. The treatments are required to facilitate regeneration, repair, and replacement of tissues. The tissue transplantation is the most common method for treatment. The transplantation can be carried out in two ways: tissue from one individual to another (allograft or transplant) or from one place to another place in the same patient (autograft). Both of these two ways have some problems: gathering autograft is uneconomic and sore. Furthermore, anatomical restrictions and donor morbidity can constrain tissue/organ transplantation. Transplants and allografts have similar problems like autografts. There is no enough tissue for sick person who needs a transplant or allograft. When carried out transplantation, a sick person can face risk of transportation of disease or infection which can pass from the donor to patient [2].

1.2. Musculoskeletal and Skin Tissue Engineering and Skin Regeneration

The skin plays an important role to protect the body from th environment. The structure, comprising of the skin layer is essential to its function–barrier to protect physically, sensation, thermoregulate, immunity, and homeostasis. The significance of those roles happens evident by the disturbances physiologically that consist of patients whom have wide chronic ulcers, or wounds and burns, or toxic epidermal necrolysis [3].

Soft tissue plays an important role in preserving contours and serves based mechanically pad in terms of muscles, skin or bones. Tissue engineering technologies are a multi-disciplinary area of biomedical and bioengineering that allows researcher for production tissue by combination a little quantity of high growing and distinction prospective cells within an artificial scaffold with growth factors [4].

Efficient repair or renewal of harmful tissue and organ relies on the matutinal regeneration of blood flow required for metabolic support and cellular infiltration. Biomaterials that are implantable designed to replace injured tissues should serve (ie, 3D tissue engineering structures) through which cells can pass and provide this required blood flow. Likewise, biomaterial polymers need to be strong sufficient to withstand the physiological requests imposed upon them after implanted in a specific organ and keep its mechanical property.

An approach for regeneration injured tissues benefits complete extracellular matrix (ECM) for host cells as growing bracket from animal tissues. It has not got cellular part of a tissue, and comprise of protein – carbohydrate structures excreted based on cells. The constituent of ECM is collagen commonly, the constructional protein. Its materials can be reference to as inherently forming scaffolds from polymer, occurring biopolymers naturally, ECM scaffolds, bioscards, biomatrics or when generated from the tissue and produced into a scaffold prosthesis. Structures are placed acellular to replace diseased or injured tissues, and host cells are removed [5].

The musculosketal system includes different supportive tissues such as bone, cartilage, muscles, tendon, ligament with skin that support the shape, stucture and protect of human body. If the tissue has been injured because of different causes like diseases, accidents and malignancies, it is required to repair and replacement with healthy tissue. The fundamental matter for tissue engineering is biomimics for organo-genesis which are successful recently. Biomaterials that are designed as 3 – dimensional (3D) structures have a significant act to regenere musculoskeletal system. Natural and non-natural biomaterials can be used for scaffold based on injured tissue types [6].

1.3. 3 – Dimensional (3D) Bioprinting

3D Bioprinting form biomaterials is a newly developing technology that purpose is inovation on organs and tissues with new developments. The technology is under research and most of researchers are acted on studies. 3D printing is a process to control cell interactions like proliferation, differantiation and migrattion within 3D bio-printed structures. For that reason, different 3D-bioprinting methods are utilized for different soft-tissue-engineering implementations [7].

3D bioprinting enables a viable and precise processing of cells with biomaterials for production. The perfect degradability, biocompatibility and processability of the gelatin-Methacrylate (GelMA) prepolymer will act a crucial role as a printable biomaterial in 3D bio-printing biotechnologies [5]. The direct 3D cell bioprinting method was used to form multilayer tissue engineering structures containing a collagen-based solutions as the structural part to mimic fibroblasts, keratinocytes and layers of skin [8].

Bioprinting is an under-class of additional production technique which involve the certain location of cells with materials except or include carrier material to reconstruct the complicated set-up of tissues with organs. The probability of compounding various cell tips and materials makes the approach exclusively curious of tissue engineering, because it may help replicate the natural regional organization of tissue. In biopression, hydrogels are mostly used as cell carriers [9].

3D printing applications have been improved in used by the gelatin-based scaffolds to manufacture 3D constructions with countless functions as biologically, chemically, physiologically, physically and clinically [10,11].

Bioprinting applications have been implemented to constitute GelMA depends on 3D structures for different tissue engineering applications. Bio-printing is a significant addition to the soft-tissue engineering toolbox, both on its own and also with other technique combinations. Porous scaffolds composed of PLGA and collagen have been appropriate for 3D tissue-engineered constructs. These determinations indicate that the tissue has the potential for proliferation in vitro 3D structures and is implemented as soft-tissue structures. The approach is suitable for engineering structures that need to particular shape and volume of each patient [4].

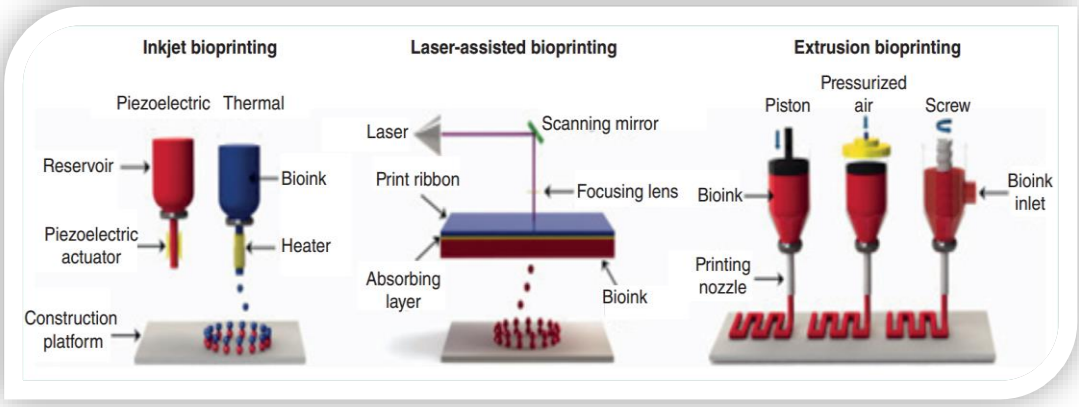


Figure 1.2. Three basic bioprinting methods [25].

Additive manufacturing is a prodution technique using 3D multi-layered structures to construct porous scaffolds of pre-designed shapes which have good osteoconductive and

mechanical features. Fused deposition model, 3-dimensional bioprinting (3D), selective laser sintering, and stereolithography are four common additive manufacturing techniques which are especially developed for tissue engineering. First 3D printing technology was developed in 1995 at MIT. 3D printing is a novel technology which is ensuring developed control over structure of fabricated scaffolds with high reproducibility given by the automatized deposition process. Nowadays, 3D printing technology is used for designing, and fabricating of scaffolds with designed shape, interconnected porosity, and controlled chemistry. 3D biprinting is a rapid prototyping technology which is using for fabricating complex structure with high precision by way of a layer-by-layer building. 3D printing which is additive and automatized process enables the manufacturing of 3D scaffolds having completely controlled architecture (internal pore geometry, external shape, and interconnectivity) with more repeatability, and reproducibility. 3D bioprinting is using for producing complex tissue structures or artificial implants by way of layer-by-layer building for specific therapy of patient. The extrusion-based 3D printing technique is similar to the fussed deposition modelling method which extrudes material from a nozzle.

In extrusion-based method, 3D printer which is equipped with pneumaticdriven or screw pistons, is utilized to force out continual cylindrical yarn of bioink from distribute tips enclosed to refill chamber. The stored layers of filaments then combine to produce the 3D structure. This contact-based printing technique uses cellular or noncellular bioinks which have a wide range of viscosities and is generally noted to be lower than inkjet and laser bioprinting approaches from the point of resolution. Additive manufacturing techniques have some advantages such as material and resource efficiency, production and design flexibility, and new business models. Small-scale production, cost, imperfections, and limited knowledge about materials and additive manufacturing process are limitations of this technology [8].

1.3.1. 3D Bioprinting for Gelatin-Based Hydrogels

Extrusion-based 3D printing technologies is emerged in different organ 3D bio-printing fields, and gelatin-based hydrogels are attracted great attention because of their excellent as physically, chemically, biologically and clinically. Gelatin-based hydrogels are manufactured as bio-inks for alien (or leak) inks for canal or pore dispositions or as solid

structures for cell lifecycle. 3D printing technologies by extrusion method are improved to use gelatin-based hydrogels for manufacturing solid 3D constructures with a huge number of functions as physically, chemically, biologically, physiologically and clinically. It may be reached that a small change in the components of gelatin-based scaffolds can modify importantly the resulting properties of the 3D bio-printed structures [10].

1.4. Natural Polymers Used in 3D Bioprinting

In wound dressing applications, natural polymers with bioactive properties are included in the structure of 3D tissue-engineered which are composed of synthetic polymers which do not contain these properties in order to increase tissue compatibility and promote cell growth and proliferation. Natural polymers are generally easily accessible and abundant in nature. In addition, they have been observed in studies where they accelerate wound healing and repair damaged tissues thanks to their structural similarities with ECM [12].

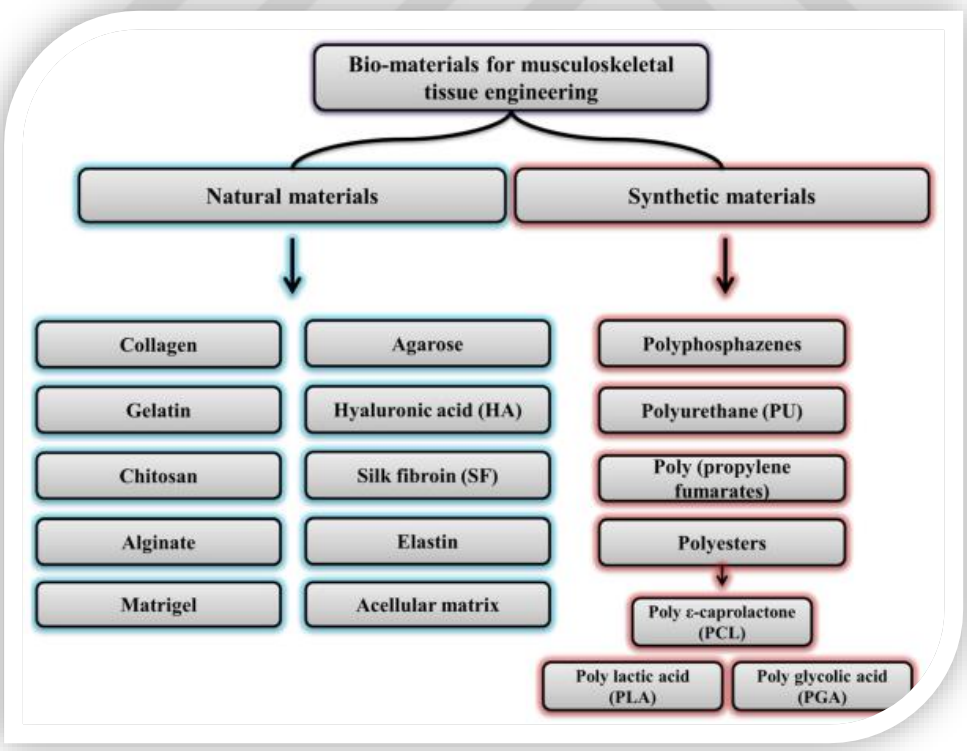


Figure 1.3. Natural and synthetic materials that in tissue engineering commonly [6]

1.4.1. Gelatin and Gelatin – Methacrylate (GelMA) Hydrogels

Gelatin is played a crucial act in biomedical material research, especially for 3D cell culture models and tissue-engineered implementation because of its biocompatibility, biodegradability and hydrogel-forming ability.

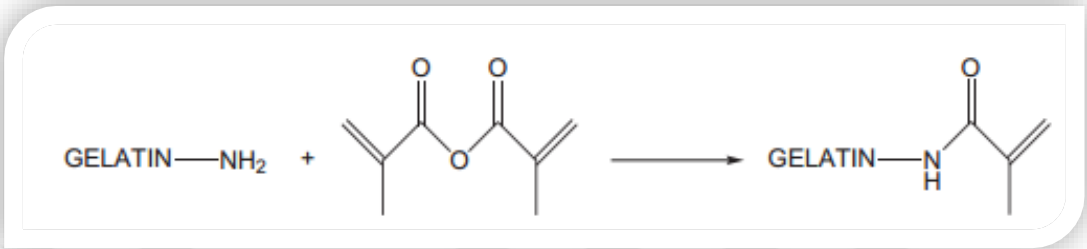


Figure 1.4. Schema for gelatin methacrylate synthesis

Gelatin as a kind of biocompatible, protein-based and biodegradable polymer is generated from degradation of collagen. It is also beneficial polymer for increasing cell surface adhesion due to have bioactive groups (L-arginine, glycine, and L-aspartic acid peptides). The solvable native of gelatin in the liquid environment at human-body temperature (~ 37° C) is one of the limitations of utilizing them in soft-tissue engineered applications, therefore it is necessary for crosslinking with agents, for examples, glutaraldehyde, lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) etc. Fibers that are crosslinking as covalent in chemically can develop gelatin mechanical features and stability [6].

Gelatin Methacrylate (GelMA) hydrogels have been demonstrated to promote distinction and chondrocyte viability due to various cross-linking parameters. It allows control over polymer density, UV exposure-period and thermic gelation, hydrogel hardness and swelling features. GelMA has lower viscosity at 37° C which is noncompatible for bioproduction applications. Nevertheless, deposition with hyaluronic acid or thermoplastics gives the use of gelMA in bioproduction process. Thoses properties can allow matching of inherent functional changes in the mechanical and geometric features of cartilage of engineering structures [10].

GelMA is exclusive by its low cost, degradability, excellent biocompatibility. GelMA is defined for 3D cell/tissue culture implementation and as a tissue-engineered applications because of these features. Though few studies that were used GelMA are issued in the recent years, compact literature search divulges considerable heterogenecity for material properties, polymerization processes used for forming hydrogels and their density [13].

Gelatin is formed mixed heterogeneous proteins, and consists of collagen that are provided by hydrolyzing in part. Collagen is also one of the most important protein in human body. For instance, there are large amount collagen in human cartilage, skin, heart, blood vessels, cornea, epithelium , tendon and ligament. Gelatin is utilized for covering of cell/tissue culture dishes to support of cell adherence of various cell tips. So it means that the gelatin is quite biocompatible material for human body. Besides, gelatin is also used in biological applications like tissue engineering and small molecule delivery. Gelatin is degraded because it has sensitive protein sequences of matrix metalloproteinase that is desired biological features in in vivo hydrogels implanted. It is based for regenerative medicine applications that are degraded - tissue-engineered structures to allow the new ECM formed deposit by cells. Chemical modification and methacrylation is happened on gelatin structure that have lysine functional groups when it is exposure to UV light.

GelMA interacts with different cell lines because of bioactive property. Due to adhesive functional groups, encapsulation of cells happens easily by GelMA. But, hydrogels made from gelatin after UV crosslinking are poor as mechanical [14]. It can be adjustable easily by changed the methacryloyl substitution degree, concentration of GelMA polymer and photoinitiator densities, and duration of UV crosslink for GelMA hydrogels and their properties [15].

1.4.2. Hyaluronic acid and Hyaluronic acid – Methacrylate Hydrogels

Hyaluronic acid (HA) is a carbohydrate group glycosaminoglycan which is naturally found in tissue ECM structure. It is a inherently consist of polysaccharide that is omnipresent in the human body and forms an significant part of the extracellular matrix. [16]. HA is a elastic bio-material and is degraded via hyaluronidase-enzyme. Besides, it is non – adhesive and non – immunogenic non-thrombogenic and non-adhesive polymer.

The molecule structure forms repetitive disaccharide portions of N-acetyl-β-d-glucosamine and β-d-glucurronic acid (*Figure 1.5*). HA is considered to be the main ECM component in various tissues like the central nervous system, cardiovascular tissues, epithelium, cartilage, ligament, vitreous fluids.

Hydrogel-based structures are widely utilized in reformative engineering research to renew degenerate and injured or defective tissues. The ability to produce a 3D sof-tissue matrix allows a controlled examination of cell to cell and cell to biomaterial effect. Hydrogels may mimic the natural extracellular matrix environment with soft constructions and huge water ingredient. Consequently, hydrogels are commonly utilized for two surfaces cultured and 3D cell studies for creation biomimetic structures. Those hydrogels may be shaped by ultra-violet photo-crosslinking of solutions including cells [14].

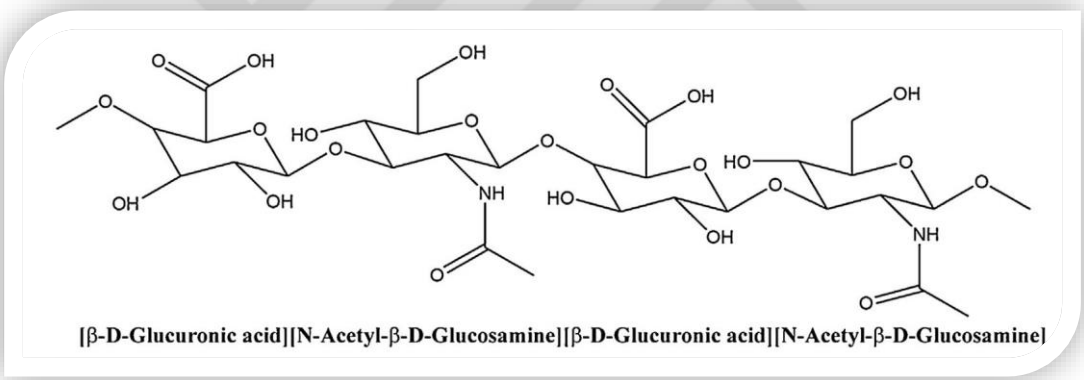


Figure 1.5. Molecular structure of hyaluronic acid.

Hyaluronic acid is a inherently occurring polysaccharide which is omnipresent in the body, also forms an important part of the ECM. As one of its diverse biological functions, HA can be defined as a very important key in the development of tissue engineering. Besides, it causes a role for wound healing and is increased in vivo tissue engineering applications [14].

Hyaluronic acid is a viscoelastic material, and it is a degredable polymer by relevant enzyme. HA is known as a component of the ECM in different tissues like cardiovascular tissues, epithelial, nervous system, cartilage, and connective besides vitreous and synovial

fluids. For that reasons, the material is reported to play crucial roles in wound healing, angiogenesis, cell-receptor interactions and proliferation [14].

HA have another properties related to modification and methacrylation chemically for ease crosslinking under UV light. The carboxylate functional groups of HA is provided the crosslink. Based on this feature, hyaluronic acid – methacrylate (HAMA) can be manufactured to produce with physical properties like pore structure, stiffness and degradation at various methacrylation degrees. For HAMA, in spite of having good properties for biological implementations, the non-adhesive property is prevented the cell spreading studies. To prevent of this, adding of functional groups that are interacted with cells, such as gelatin, can be improved [14].

1.5. Crosslinking Process for Hydrogel

Crosslink by UV lights is an initiative for stimulation the formation of 3D hydrogel links. It provides several advantages when compared to other inducements, for example, photocrosslinking is most useful, simple and fast route of manufacturing 3D hydrogels with controllable spatial resolution, structures and dimensions. Cell laden hydrogels after photocroslinking is used to with success in most biological and medicinal applications, like regenerative medicine, soft and hard tissue engineering, growth factor for understanding of cell behaviour such as differantiation, migration, proliferation and endothelialization.

Manufacturing of hybrid hydrogels is a state-of-art study that helps to development of the material and biological features of biomaterials. In spite of they are biomimetic substrates, material features of HAMA and GelMA hybrid hydrogels are not characterized completely [14].

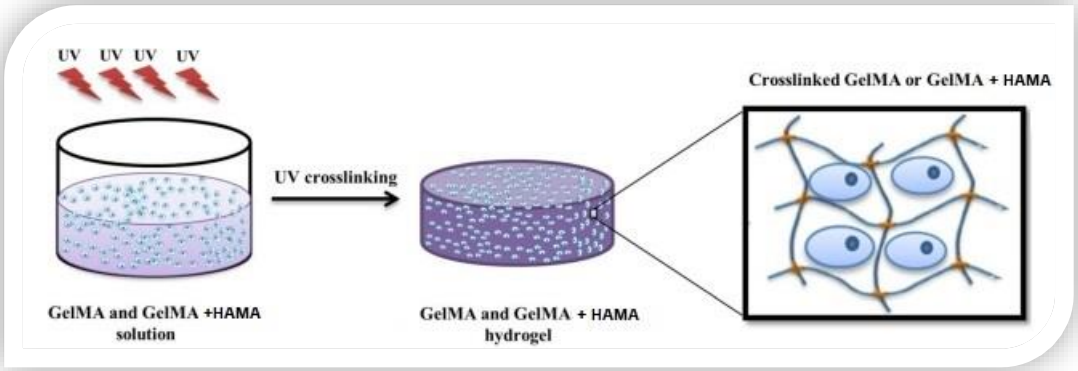


Figure 1.6. Preparing of GelMA and GelMA+HAMA hydrogels for soft tissue engineering

2. MATERIAL AND METHOD

2.1. Materials and Equipments

2.1.1. Materials used for gelatin – Methacrylate synthesis

- Gelatin (Type A, porcine skin; Type B, bovine skin from Sigma)
- Methacrylic anhydride (Aldrich)
- Phosphate buffer saline (pH 7.4; @25° C; ChemBio)

2.1.2. Material used for hyaluronic acid – Methacrylate synthesis

- Hyaluronic acid (Sigma)
- Methacrylic anhydride (Aldrich)
- Phosphate buffer saline (PBS) (pH 7.4; @25° C; ChemBio)
- Sodium hydroxide (Sigma – Aldrich)

2.1.3. Materials used for dialysis

- Dialysis tubing cellulose membrane (Sigma)
- Distilled water (M_w=18.00 g/mol, H_2O from YasinTeknik, Turkey)

2.1.4. Material used for hydrogel forms

- Photoinitiator – LAP (lithium acylphosphinate salt)
- Phosphate buffer saline (pH 7.4; @25° C; ChemBio)

2.1.5. Equipments

- Micropipette (100-1000 µL, Brand, Germany),
- Needle tips (with 22 and 23 gauge, AliExpress, China),
- Microscope slides (26x76 mm, 1.0 mm – 1.2 mm thick, Isotherm, Turkey),

- Vortex mixer (0-3000 rpm speed, Four E' S Scientific) was used for dissolution of materials in solvents.

- The magnetic stirrer (Hei-standard, from 100 to 1400 rpm and temperature setting up to 300° C, Heidolph, Germany) was used for preparation of gelatin with methacrylic anhydride (GelMA) solution.

- Precision weighing (XB 220 A, Precisa, Germany) was used for weighing of materials.

- pH meter (pH7110, InoLab) was used for pH measurement of solutions.

- Incubator (Ecocell, MMM Group Medcenter Einrichtungen GmbH) was used for dialysis process at 37° C.

- A freeze – dryer (CoolSafe – ScanVac, Denmark) was used for lyophilization of the solutions.

- An Ultraviolet Light Lamp (UVP – UVGL-58 Hangheld UV Lamp, Upland, CA, USA; 6 Watt, 254 / 365 nm; 0,16 Amps; 230 V ~ 50 – 60 Hz) was used to crosslink the samples on hydrogel preparation step.

- Pycnometer (with 10,265 mL volume, Borucam, Turkey) was used for density determination of solutions.

- Tensiometer (Sigma 703D, Biolin Scientific) was used for surface tension measurement of solutions.

- Viscometer (DV-E, Brookfield) was used for viscocity of solutions.

- The Ultimaker 2 + 3D printer (Ultimaker B.V.) is used for manufacturing of 3D soft tisuue engineered structures.

- Swelling and biodegradation test were performed by using a Thermo-Shaker (TS – 100C, BioSan, Riga, Latvia)

- Mechanical properties (tensile strength) of 3D engineered structures were determined using (Shimadzu EZ-LX). Digital micrometer (293-100, Mitutoyo, Japan) was also used for measurement of cutted structures' thickness.

- Scaning electron microscopy (EVO MAA-10, Carl Zeiss, USA) was used to image the morphological properties of tissue skeletons.

- Chemical characterizations of 3D soft tissue structures were made by Fourier transform infrared spectroscopy (FTIR, 4600 Jasco, Japan).

2.2. Methods

2.2.1. Gelatin – methacrylate synthesis

For dissolution of gelatin 10% (w/v) in PBS solution, a vortex mixer was used for a while and the solutiion was prepared with magnetc stirring at 50° C for 30 minutes. Then, the methacrylyc anhydridde was added to the solution as 0.6 times the weight amount of gelatin. After a hour of reaction at 50° C with magnetic stirrer, the mixture was dialyzed for 72 hours in distiled water at 37° C in incubator. The productt formed was freeze-dried to obtain a white solid [15, 17, 18, 26].

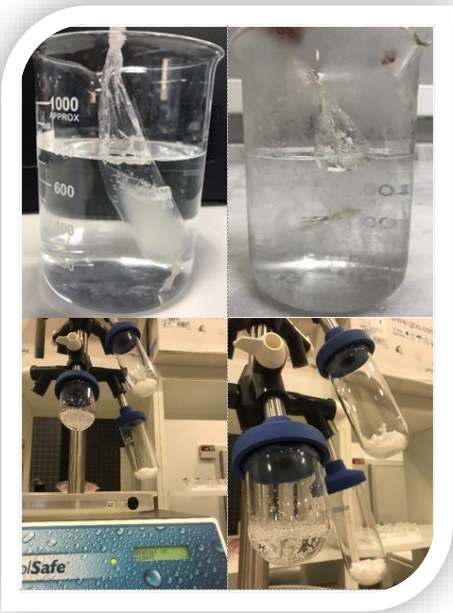


Figure 2.1. Dialysis and freeze-drying of the solutions

2.2.2. Hyaluronic acid – methacrylate synthesis

For dissolution of one gram hyaluronic acid in 100 ml distilled water, a vortex mixer was used for a while and the solution was prepared with magnetic stirring until homogenization of solution. Then, the methacrylic anhydride solvent was added to the solution at 1(v/v) and then the reaxtion was started. It was maintained the pH between 8-10 by adding of 5M NaOH (sodium hydroxide) for a day. After 24 hour of reaction at 4° C, the solution was dialyzed for 72 hours in distilled water at 4° C. The product formed was lyophilized to obtain a white solid product for 3 days [14, 17, 18, 27].

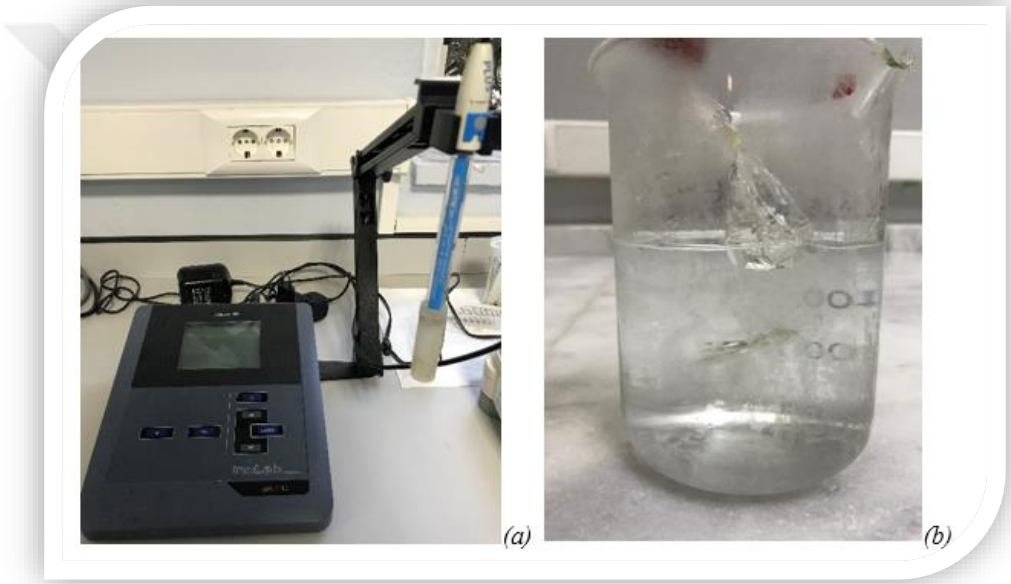


Figure 2.2. (a) pH meter; **(b)** Hyaluronic acid – methacrylate solution in distilled water during dialysis

2.2.3. Preparation of hydrogels

Two hydrogel pre-solutions were prepared as shown in *Table 1*. The pre – polymers (HAMA and GelMA) were measured at 10% w/v GelMA and HAMA concentrations. All hydrogel precursor solutions were dissolved in PBS. Then, it was also measures 0.5% w/v LAP concentration, and added to pre-polymer solutions, and mixed them in PBS at 50°C until all was dissolved [28].

Table 1. Composition of hydrogels used in thesis.

Compositions (% w/v)		**Nomenclature**
10% GelMA		Gelatin methacrylate solution (GelMA)
9.5% GelMA	0.5% HAMA	Gelatin-methacrylate & Hyaluronic acid-methacrylate solution (GelMA + HAMA)

Before crosslinking by UV light, the solutions were in a incubator at 37° C. The solutions containing GelMA is light sensitive; was maintained lyophilized GelMA in the dark, like by wrapping falcons in aluminum foil.

2.2.4. Physical characterization of hydrogels

The solutions (GelMA and GelMA&HAMA) were tested to determine characterization properties.

2.2.4.1.Density measurement

A pycnometer was used for measurement of density property. After preparation of solutions, approximately 10 ml solutions were poured into the pycnometer. Then, the solution-filled pycnometer was re-weighed. The results was recorded.

2.2.4.2. Surface tension measurement

Tensiometer (Sigma 703D, Biolin Scientific) was used for surface tension measurement of solutions. The surface tension of the solution put into a glass beaker was measured according to the instructions of the device and the value was recorded.

Figure 2.3. Measurement of surface tension properties of solutions by tensiometer

2.2.4.3.Rheological measurement

The viscosity of solutions (GelMA and GelMA+HAMA) was specified at room temperature by a viscometer (DV-E, Brookfield). The parameters were set up for appropriate value of the solutions according to viscosities.

Figure 2.4. Measurement of rheological properties of solutions by viscometer

2.2.5. Bioprinting of hydrogels

The prepared hydrogels (GelMA and GelMA+HAMA) were printed by using Ultimaker 2 + 3D printer. The structures were printed on petri dishes. There are several parameters on machine. They were optimized to best 3D soft tissue engineered products. The parameters with ranges were given on table below.

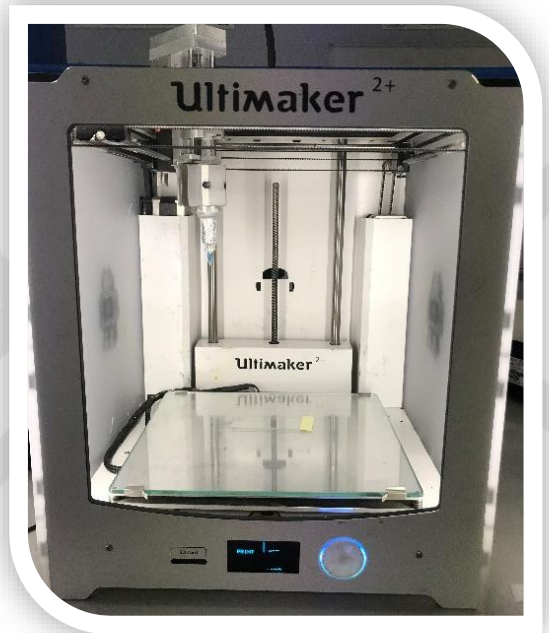


Figure 2.5. Printing of the soft tissue engineered construction by the 3D Bioprinter

Table 2. Parameters of 3D Bioprinter during optimization

Parameters	**Values**
Number of layers	10 – 15
Nozzle diameter (mm)	0,50
Needle tip diameter (mm)	0.711 and 0.609
Extrusion multiplier	0.5 – 1,25
Extrusion width (mm)	0,40
Infill rate (%)	20 ~ 40 %
Default printing speed (mm/ min)	750,0

2.2.6. UV croslinking of 3D reconstructions

Firstly, the UV (Ultraviolet Light Lamp, 365 nm) crosslink for 3D construct was started during printing process for 10 second after 2 layers were printed. After completion of the printing of 3D constructions, exposure to UV light was continued for 120 seconds, and then UV light exposure was ended.

Figure 2.6. UV light for cross-linking of 3D reconstructions

2.2.7. Morphological analysis of 3D reconstructions

Dimensional and morphological analyzes of the 3D printed scaffolds were characterized using optical microscope (BX51M model, Olympus, Japan) and scanning electron microscopy (SEM, EVO MA-10, Zeiss, Germany).

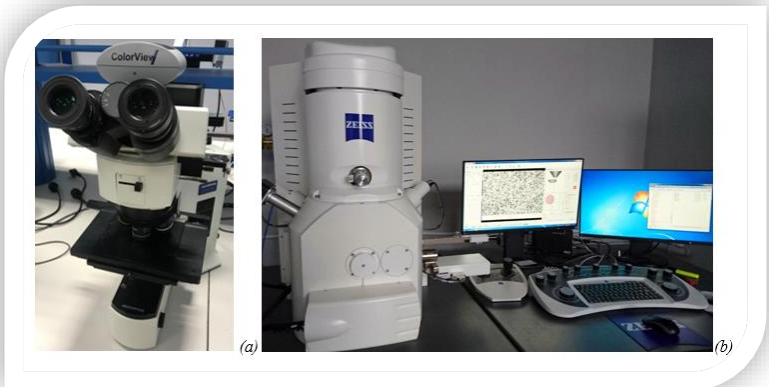


Figure 2.7. Visualization of 3D bioprinted constructures by **(a)** Optical microscope and **(b)** scanning electron microscope (SEM).

2.2.8. Chemical Analysis 3D reconstructions

The existence of chemical bonds of both solutions before printing and GelMA and GelMA + HAMA constructions after produced with 3D bioprinter were analyzed by Fourier Transform Infrared Spectroscopy (FTIR – 4700, Japan). The wavelength value of FTIR device ranges between 450 and 4000 cm^{-1}.

Figure 2.8. Analysis of chemical bonds of 3D bioprinted constructures by FTIR spectrometer

2.2.9. Determination of thermal and mechanical properties of 3D reconstructions

Thermal properties of the scaffold were analyzed using differential scanning calorimeter (DSC) (DSC-60 Plus model, Shimadzu, Japan) device in the closed pan which shows the thermal transitions of the structures. Heating temperature ranges were adjusted from 25° C to 200° C and the heating rate was adjusted to 10° C / min.

Figure 2.9. Thermal property of the 3D soft-tissue engineered structures were analyzed using DSC.

Tensile strenghth test was conducted to determine the mechanic characteristics of the produced 3D soft tissue engineered structures. The displacement strain and displacement stress values were measured with this study. The speed of test was adjusted 5 mm/min and two samples were chosen for each concentration to get precise value.

Figure 2.10. Mechanical property of the 3D soft-tissue engineered structures were analyzed using tensile test machine.

2.2.10. Swelling ratio and degredation tests

In order to prepare the samples for swelling ratio (X), three pieces of three different 3-dimensional structures, approximately the same dry mass ($W_{1,dry}$), were cut and placed in epandorf tubes containing 1 ml of PBS. They were was left in thermoshaker for 24 hours at 37° C and 250 rpm. At the end of 24 hours, the wetness of the swollen structures were gently removed by means of filter paper and mass weighed. The swelling rate was calculated by dividing the wet weiight (W1) by dry weight and the number obtained was converted to the correspondent percentage (%).

$$X = \frac{W_1 - W_{1,\,dry}}{W_{1,\,dry}} \times 100 \qquad (1)$$

The 3D structures for degradation test (Y) were conducted as mentioned above for the swelling rate study. Initial weights ($W_{2,dry}$) were recorded. Dried structures were then incubated on a shaker with 250 rpm at 37° C, their degradation was recorded at different time period (1, 4, 6, and 24 h). The percennt mass remaining by degradation (W_2) was analysed by dividng the dry weight with the structure weight that weighted firstly, the results then were converted into correspondent % values.

$$Y = \frac{W_2 - W_{2,\,dry}}{W_2} \times 100 \qquad (2)$$

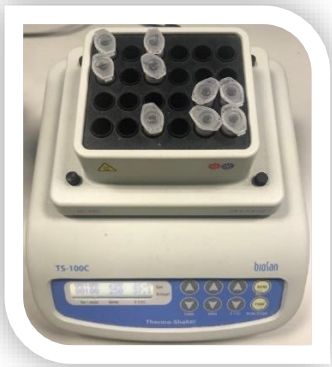


Figure 2.11. Swelling and degradation tests were performed by using Thermo-Shaker device.

2.2.11. Cell culture for 3D reconstructions

Live and dead cells were visualized Scanning Electron Microscopy, respectively, at day 1, 3 and 7th. The 3D printed structures were transferred to 24-well plates and sterilized by UV light for a night, and then, before cell seeding, samples were incubated in medium for 2 hours. Fibroblasts obtained were cultured onto structures in monolayer cultures using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-amphotericin for specific intervals at 37° C with 5% CO_2, after that the examination of cell viability and adherence.

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test was conducted for cell viability after 1, 4 and 7 days of incubation periods on 3D printed structures. Before addition of MTT solution at the concentration of 0.5 mg/mL and incubated for 5 h at 37 °C with 5% CO_2, the samples were washed with cold PBS (pH 7.4). Based on the manufacturer's instructions, the supernatant was then removed quietly followed by the addition of 1.5 mL of dimethylsulfoxide (DMSO). Plates were also incubated for 15 min with 5 % CO_2 at 37° C, and the absorbance value was measured in 590 nm wavelength using a microplate reader. Morphological analyzes of the 3D printed scaffolds were characterized using scanning electron microscopy. After 7 days of cultivation, samples were fixed by 2.5% glutaraldehyde (Sigma, USA) for 2 hours and dehydrated ethanol.

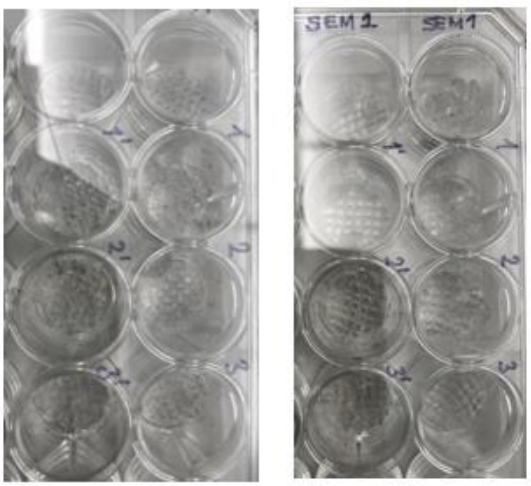


Figure 2.12. Cell culture of 3D structures.

3. RESULTS AND DISCUSSION

3.1. Characterization of Hydrogels

3.1.1. Density measurement

It was detected that the density value of gelatin-methacrylate were decreased when the hyaluronic acid-methacrylate solution was added as defined in *Table 3*. 1.05 g/mL for GelMA solutions and 0.85 g/mL for GelMA+HAMA were density values.

Table 3. Density results of solutions

Solutions	**Density Results**
GelMA (g/ml)	1,05
GelMA + HAMA (g/ml)	0,85

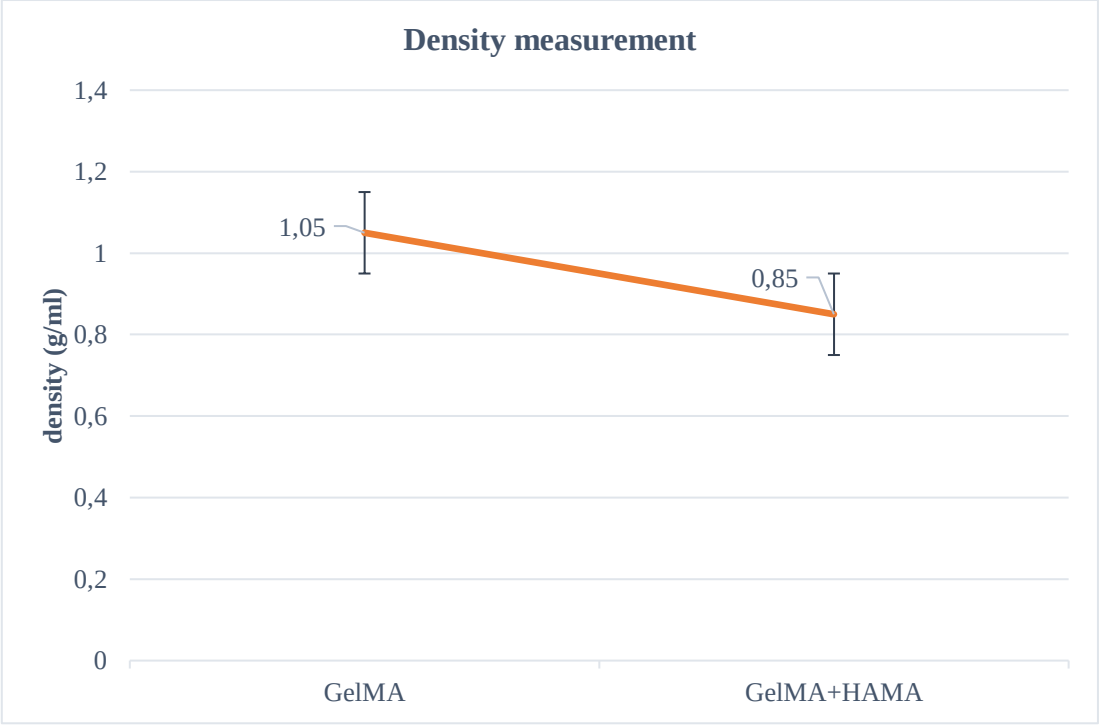


Figure 3.1. Comparison of GelMA and GelMA+HAMA solutions in terms of density values

3.1.2. Surface tension measurement

As shown in *Figure 3.2*, 26.80 mN/m for GelMA and 22.86 mN/m for GelMA+HAMA were average values of surface tension. Both the density and the surface tension of the solutions were found to decreased when added hyaluronic acid-methacrylate. The results are given on *Table 4* below:

Table 4. Surface tension results of solutions

Solutions	**Surface Tension Results**	**Average value**
GelMA (mN/m)	n=1; 28,30	26,80
	n=2; 27,42	
	n=3; 24,80	
GelMA + HAMA (mN/m)	n=1; 21,35	22,86
	n=2; 23,42	
	n=3; 23,82	

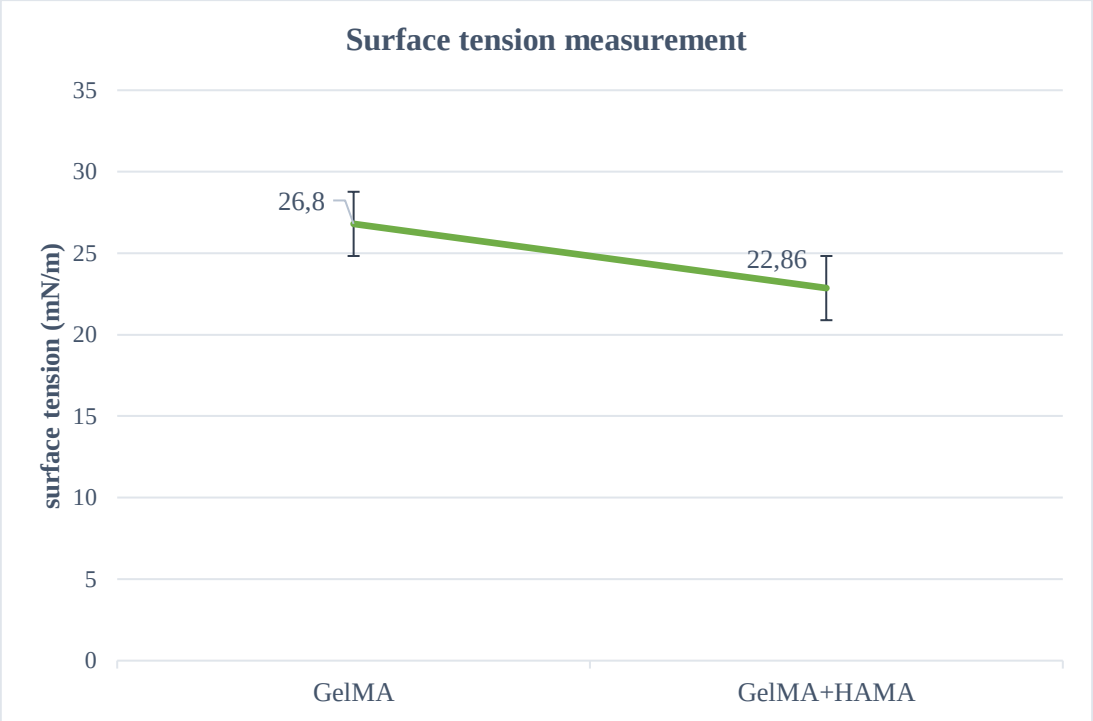


Figure 3.2. Comparison of GelMA and GelMA+HAMA solutions in terms of surface tension values

3.1.3. Rheological measurement

252.2 mPas for GelMA and 723.3 mPas for GelMA+HAMA were viscosity values as shown in *Figure 3.3*. In the comparison of viscosity values, it was also found that the viscosity was more dense than the HAMA containing solution. Since no measurement could be taken at a low rpm rotation speed, it was increased to 6 rpm and the results were recorded. When the literatures are reviewed, the results for GelMA solution are match [19, 17]. The results are given on *Table 5* below:

Table 5. Viscosity results of solutions

Solutions	**Viscosity Results**	**Average value**
GelMA (mPa s) (@ 6 rpm)	n=1; 249,3	252,2
	n=2; 251,4	
	n=3; 255,9	
GelMA + HAMA (mPa s) (@2 rpm)	n=1; 690	723,3
	n=2; 721	
	n=3; 759	

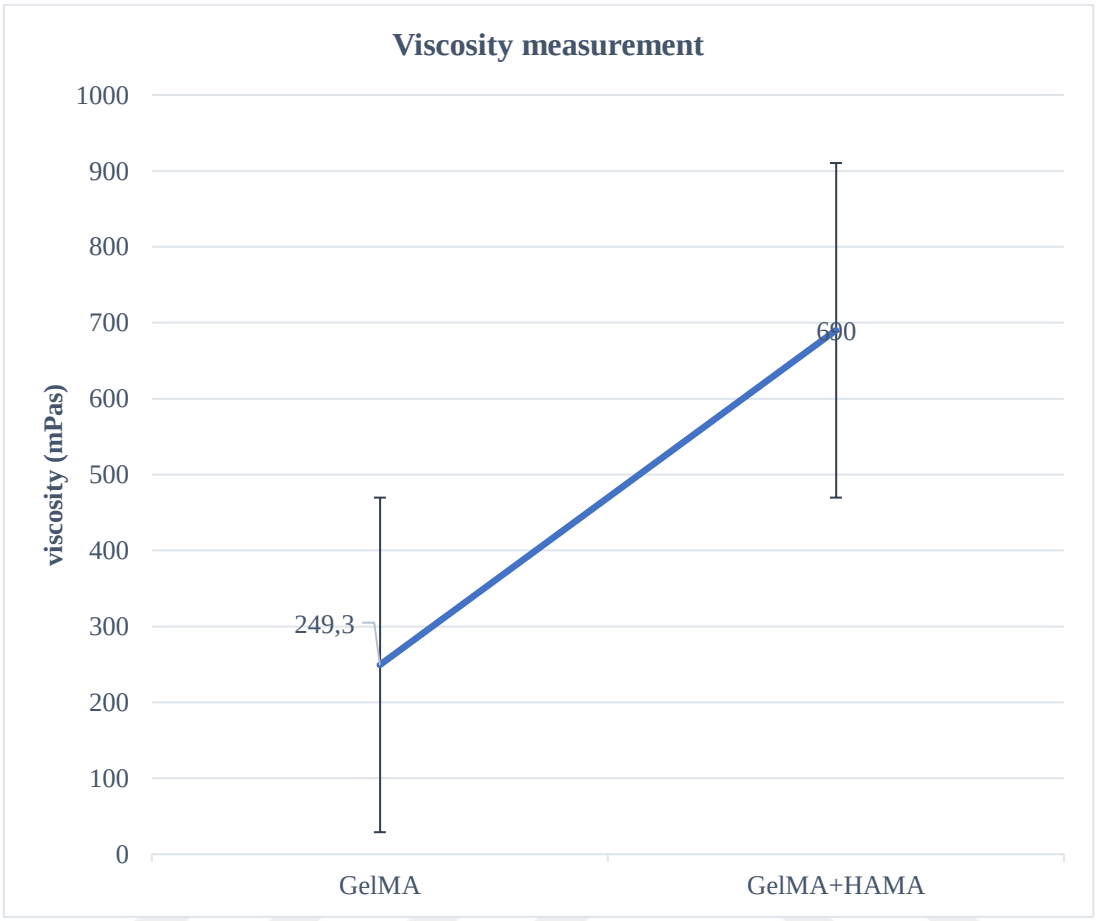


Figure 3.3. Comparison of GelMA and GelMA+HAMA solutions in terms of viscosity values

3.2. Optimization of 3D Bioprinting with Morphological Analysis

After preparation of solutions, the hydrogels were printed with different variants like infill and extrusion multiplier. The values are changed between 20 to 40% for infill ratio, and from 0,5 to 1,5 for extrusion multiplier ratio.

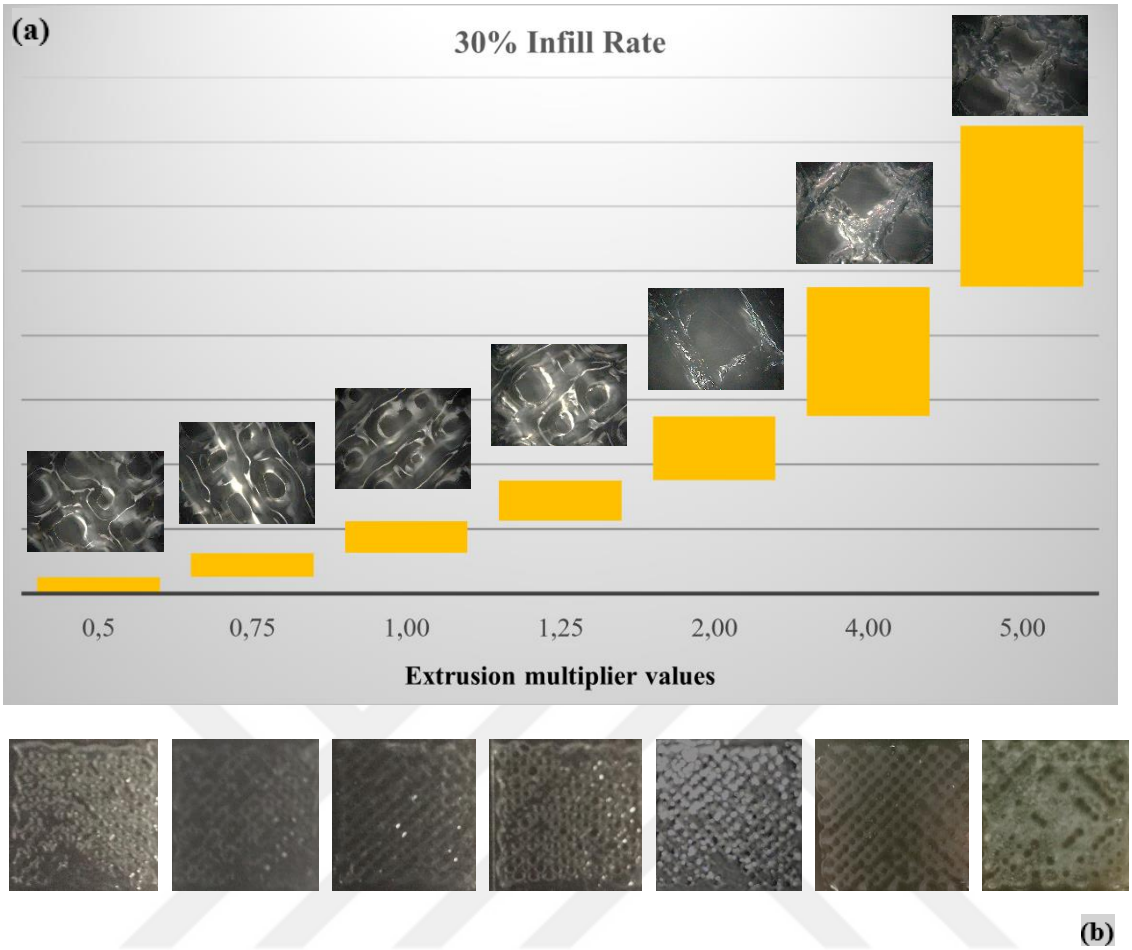


Figure 3.4. (a) The extrusion multiplier values at 30% infill rate for GelMA structures; **(b)** Images of GelMA structures for same parameters, respectively.

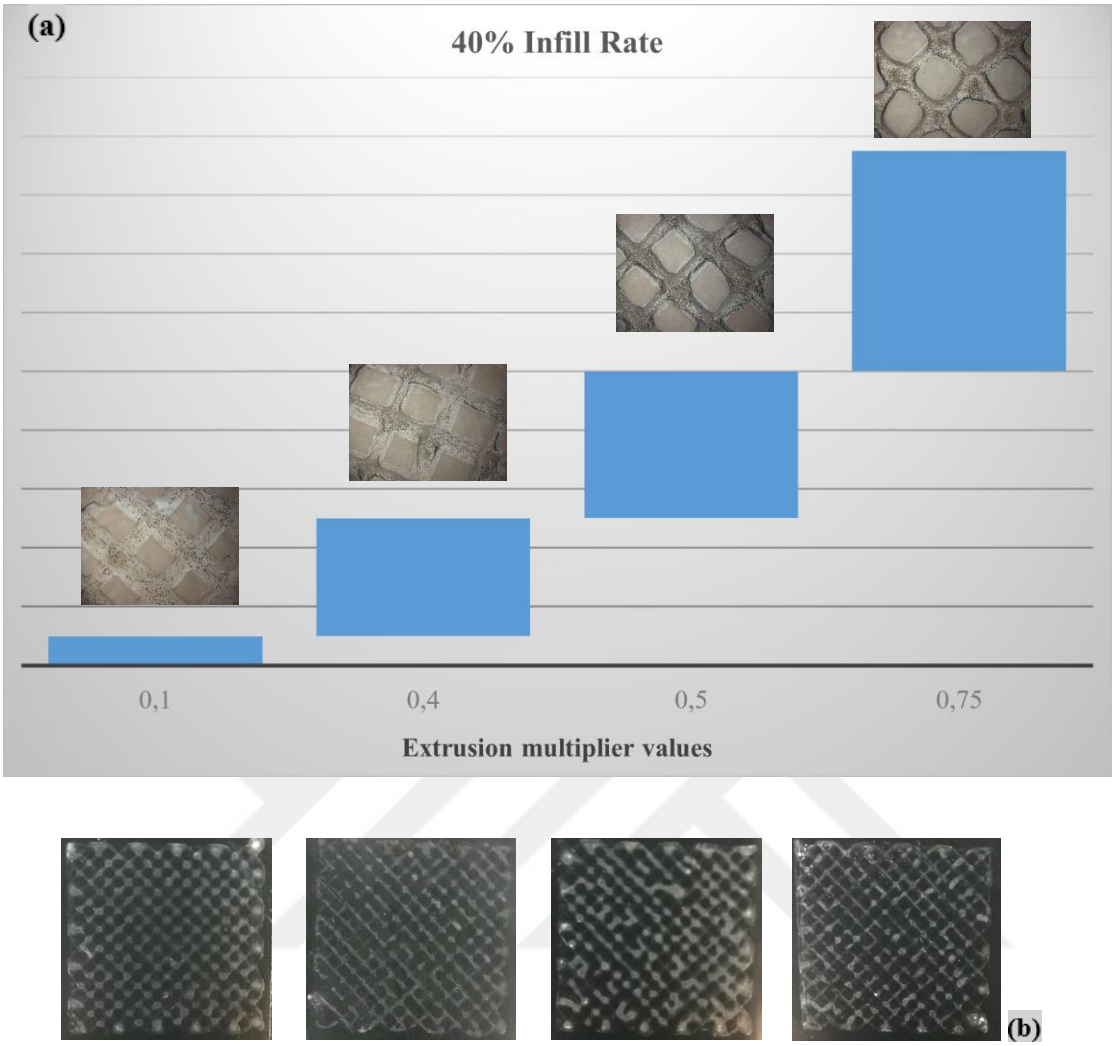


Figure 3.5. (a) The extrusion multiplier values at 40% infill rate for GelMA+HAMA structures; **(b)** Images of GelMA+HAMA structures for same parameters, respectively.

After optimization studies, it was decided that the best values were for 20% infill rate in terms of both visual and printability. As defined in *Table 6*, the values are stabilized at 20% infill rate for both hydrogels. Extrusion multiplier is changed, the value is 1.25 for GelMA, and 0.75 for GelMA+HAMA hydrogels.

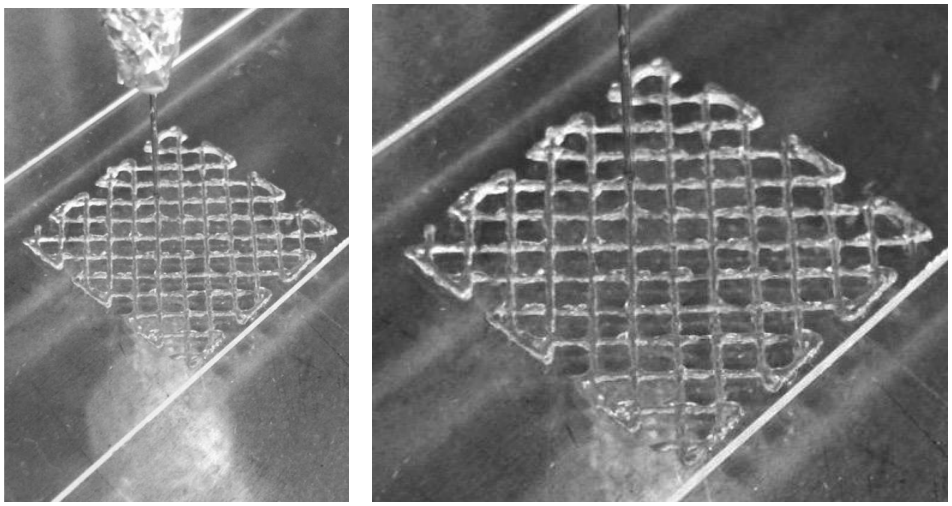


Figure 3.6. Bioprinting of 3D tissue engineering structures

Table 6. 3D bioprinting values for GelMA and GelMA+HAMA solutions

Parameters	**Values for GelMA**	**Values for GelMA + HAMA**
Number of layers	10	15
Nozzle diameter (mm)	0,50	0,50
Needle tip diameter (mm)	0.711	0.609
Extrusion multiplier	1.25	0.75
Extrusion width (mm)	0,40	0,40
Infill rate (%)	20 %	20 %
Default printing speed (mm/ min)	750,0	750,0

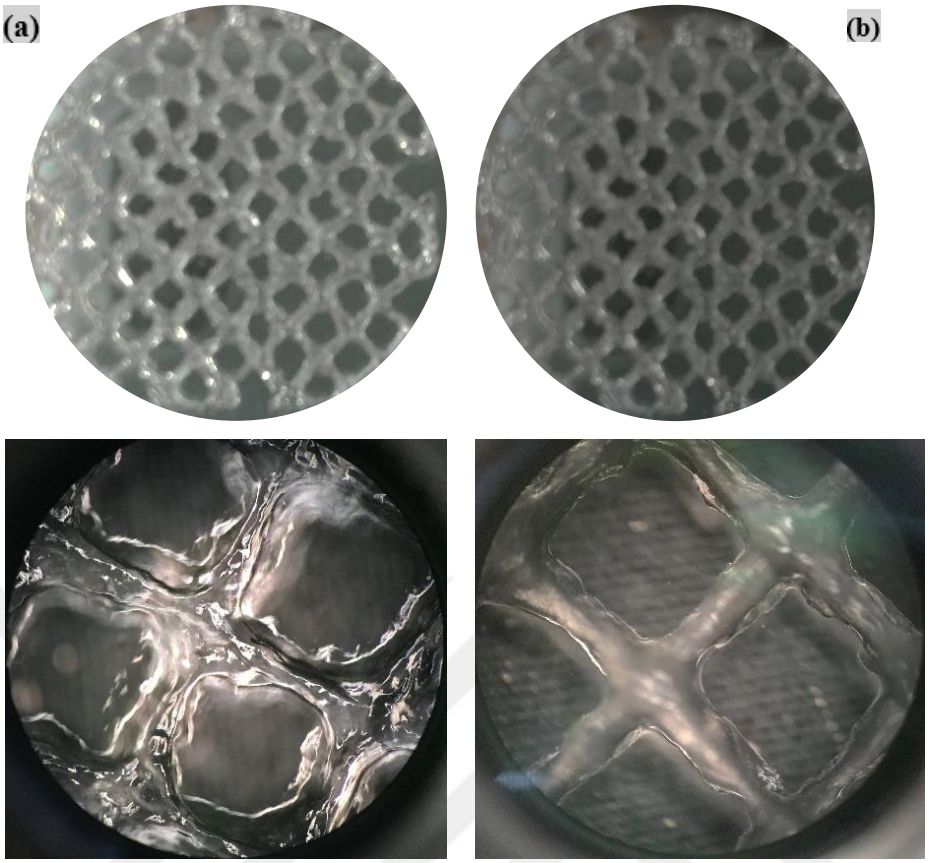


Figure 3.7. (a) Images of GelMA 3D structures at 20%infill ratio and 1,25 extrusion multiplier; **(b)** Images of GelMA+HAMA 3D structures at 20%infill ratio and 0,75 extrusion multiplier with optical microscopy at 5x.

Scanning electron microscopy (EVO MA-10, Carl Zeiss, USA) was used to image the morphological properties of 3D tissue structures manufactured from GelMA and GelMA+HAMA as following *Figure 3.8* and *3.9*.

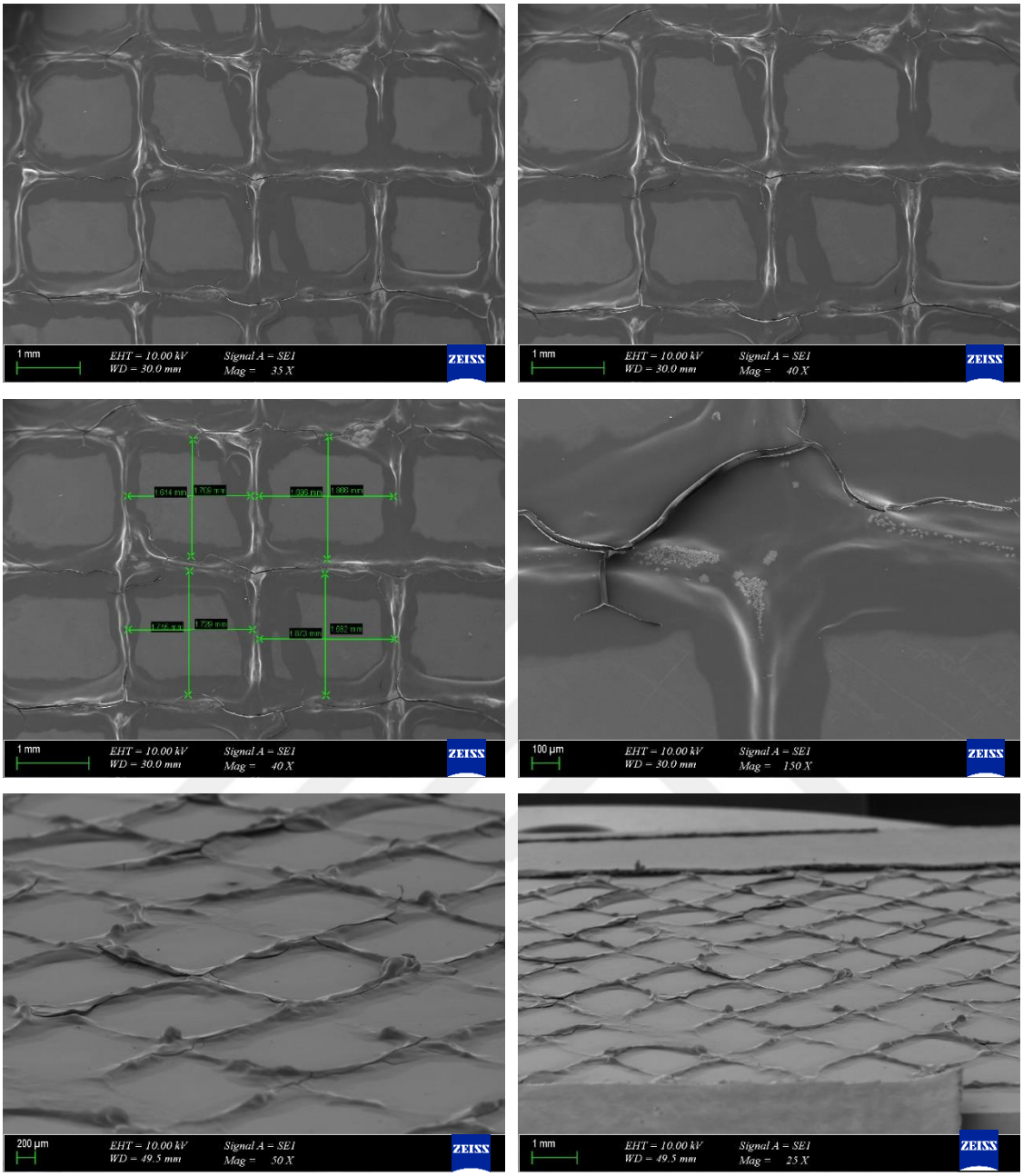


Figure 3.8. SEM image of GelMA 3D reconstructions with 20% infill rate from surface at 29x, 40x, 40x, 150x, 50x and 22x, respectively.

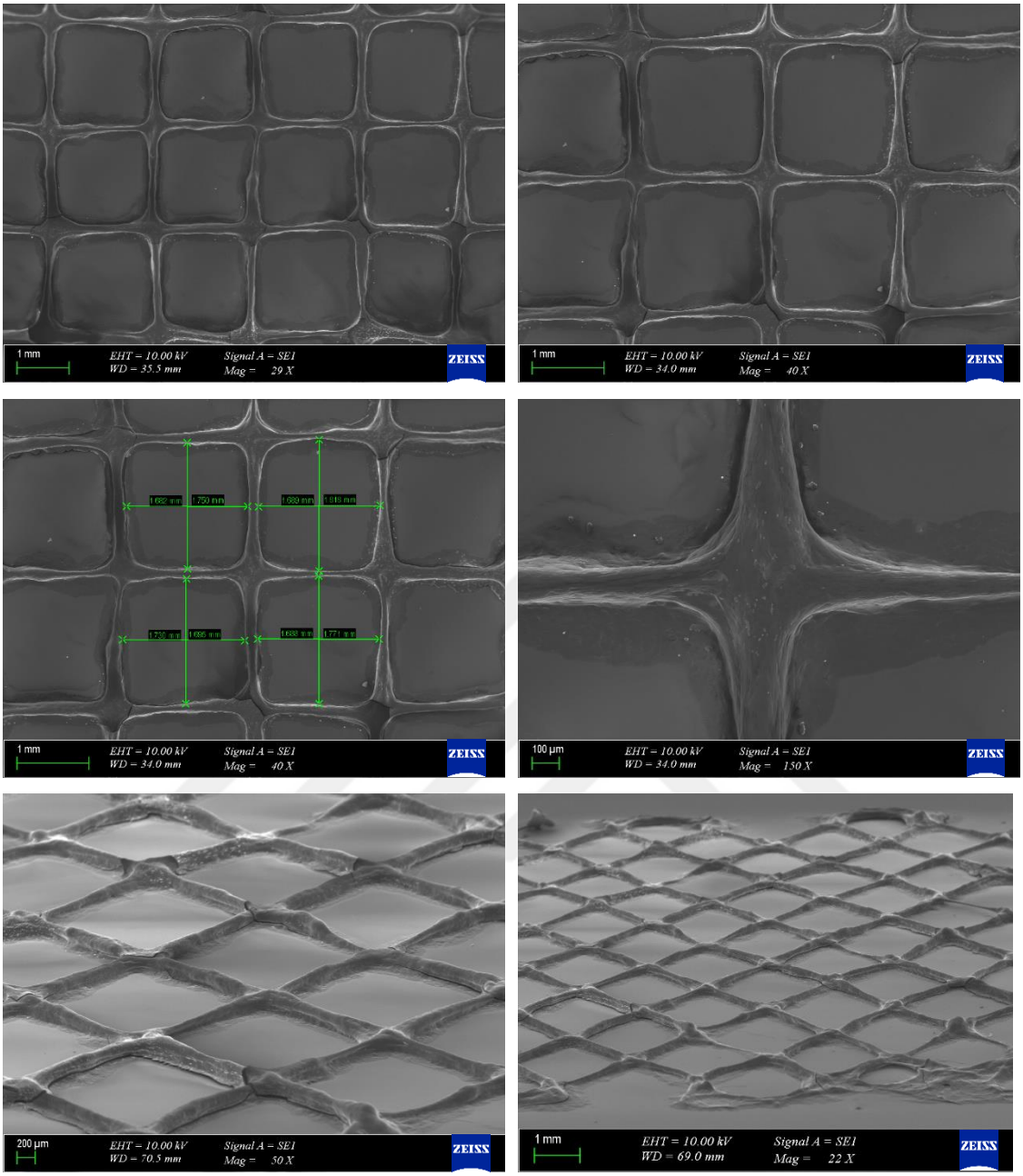
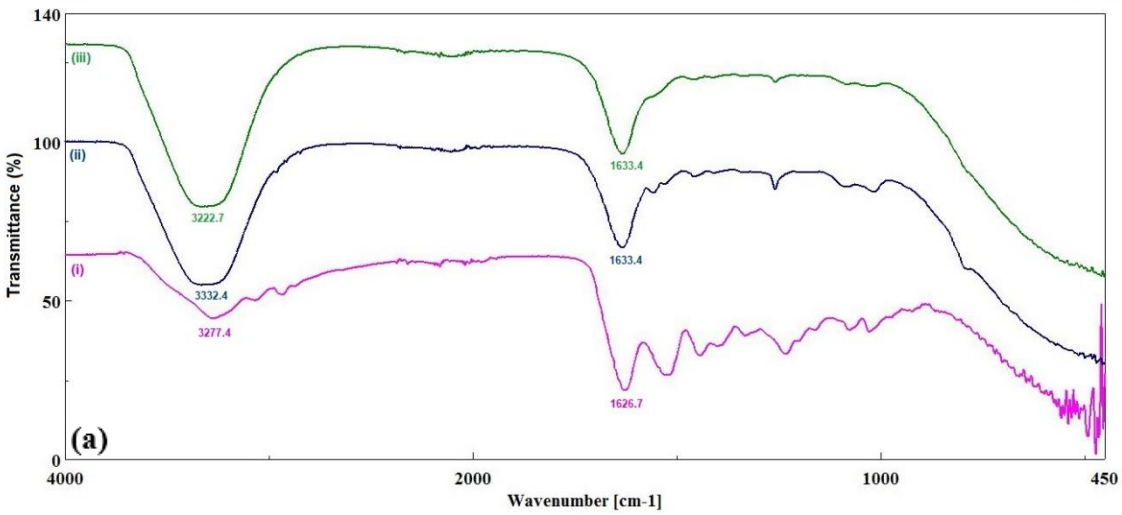


Figure 3.9. SEM image of GelMA+HAMA 3D reconstructions with 20% infill rate from surface at 29x, 40x, 40x, 150x, 50x and 22x, respectively.

3.3. Chemical Analysis

FTIR is an important technique to investigate the molecular and structural properties of polymers. The functional groups related to pure gelatin and their hydrogels with methacrylic anhydride and hyaluronic acid-methacrylate were identified by FTIR spectroscopy both before and after printed. As seen *Figure 3.10.*, pure gelatin had main absorption peaks at ~ 3277.4 cm^{-1}, GelMA had at ~3332.4 cm^{-1}, and GelMA+HAMA had at ~ 3222.7 cm^{-1} before printed. The other spectrum of pure gelatin showed peak around ~ 1626.7 cm^{-1}; GelMA showed at ~ 1633.4 cm^{-1}, and GelMA+HAMA showed at ~ 1633.4 cm^{-1} before printed.

When the peaks were reviewed after printed, the first peak was shown at ~ 3305.4 cm^{-1} for GelMA and ~ 3297.7 cm^{-1} for GelmA+HAMA 3D structures. Other peak was seen at around 1630 cm^{-1} for both structures made from GelMA and GelMA+HAMA. The literatures are shown same peaks at same wavenumbers with given spectrums in *Figure 3.10.* [20].



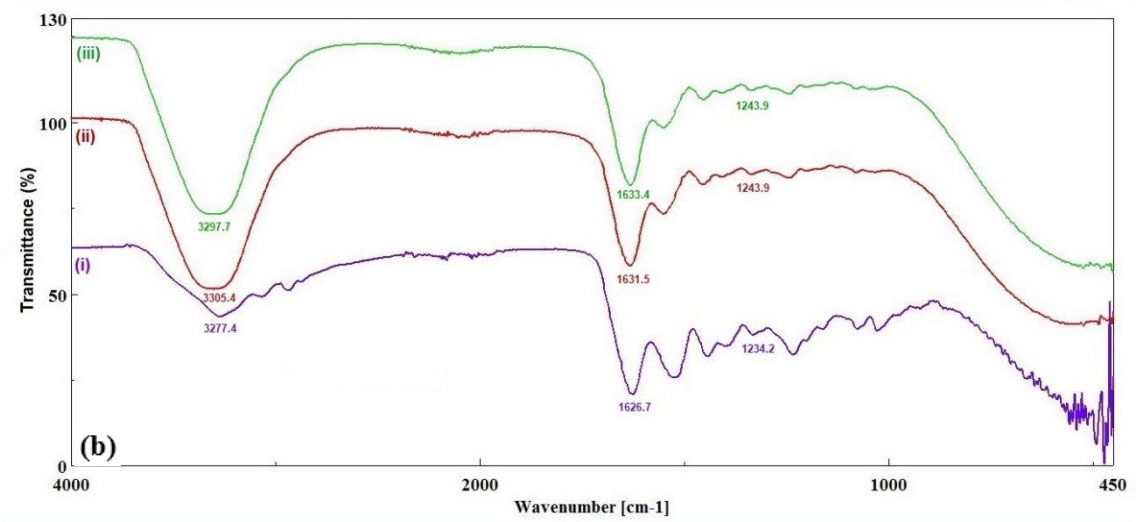


Figure 3.10. (a) FTIR spectrums of (a,i) pure gelatin, (a,ii) GelMA and (a,iii) GelMA+HAMA hydrogels before printed;
(b) FTIR spectrums of (b,i) pure gelatin, (b,ii) GelMA and (b,iii) GelMA+HAMA hydrogels after printed.

3.4. Thermal and Mechanical Properties of 3D Structures

DSC thermograms were shown for GelMA and GelMA+HAMA 3D structures in Figure 3.10. GelMA structures displayed one exothermic peak at 46.47° C. GelMA+HAMA structures displayed two exothermic peaks. First one was at 55.58° C, and second one demonstrated exothermic falling at 192.92° C. As concluded in the literature, the exothermic peak at approximately 50 - 60° C is assigned to protein denaturatiion and less-ordered states [21, 22]. Adding HAMA affects protein impurities, it causes increasing of denaturation resistance as comparison with GelMA. When the second exothermic falling at 192.92° C of the GelMA+HAMA structures is reviewed, this is represented to water loss and thermal degradation. These conclusions agree with the values which are reported in literatures [23].

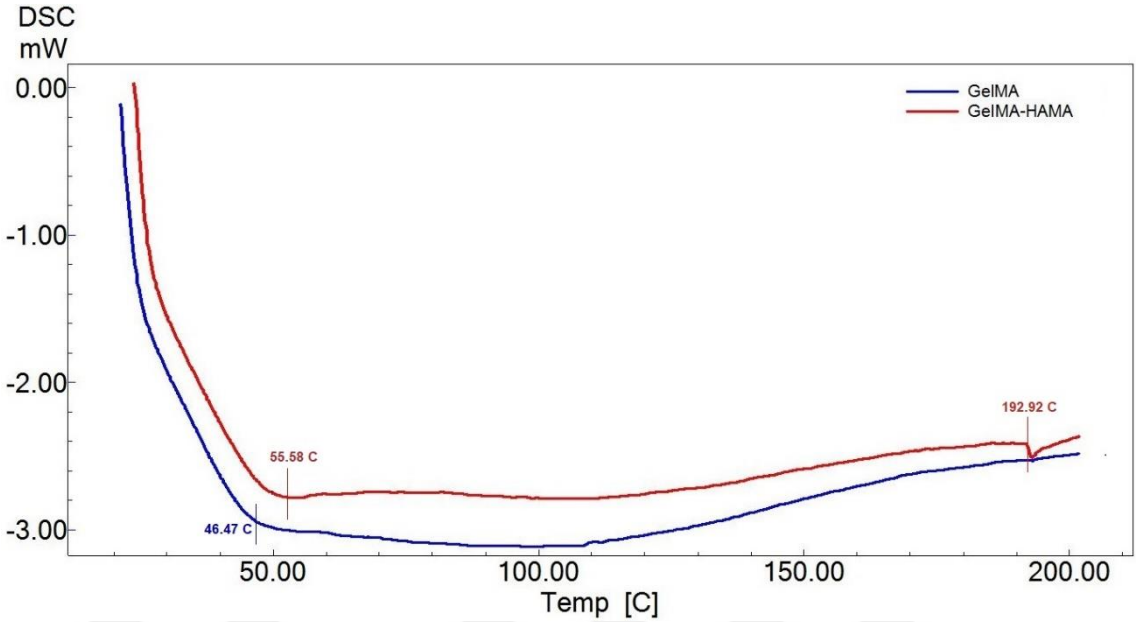


Figure 3.11. DSC thermogram of GelMA and GelMA-HAMA 3D structures.

The material stiffness increases as the polymer density enhance, which concluded an enhance in the mechanic characteristics. It was observed that the same results in our experiments as supported by other literatures [14, 22, 24]. To investigate the structural integrity of the GelMA and GelMA+HAMA 3D bioprinted scaffolds, the tensile strength device was used. As mentioned on *Table 7,* the tensile strength were determined to be 0,0240 MPa, and 0,0103 MPa for both GelMA structures, respectively. The elongation at break were determined to be 59.24%, and 51.41% for both GelMA structures, respectively. As given on *Table 8,* the tensile strength were determined to be 0,0207 MPa, and 0,0012 MPa for both GelMA+HAMA structures, respectively. The elongation at break were determined to be 78.97%, and 94.10% for both GelMA+HAMA structures, respectively. Their mechanical properties of both GelMA samples had the highest tensile strength (average 0,0171MPa) however and the smallest elongation at break (average 55,33 %) values which point out good deformability and flexibility [14, 22, 24]. By adding 1% HAMA into the GelMA solution, tensile strength value decreased but strain at break increased significantly to the value of 86.54 %. As a result of this situation, it could be said that the blending of HAMA with GelMA did not increase the strength value. This might be due to the weak physical interactions between the chains of blending polymers under loading [14]. Contrary to expectations, the addition of HA did not

improve the mechanical properties.

Table 7. Tensile strength test results for GelMA and GelMA+HAMA 3D structures

Samples	**Max. Displacement Stress (MPa)**	**Average**	**Max. Displacement Strain (%)**	**Average**
GelMA (1)	0,0240	0,0171	59,2388	55,3278
GelMA (2)	0,0103		51,4168	
GelMA+HAMA (1)	0,0207	0,0110	78,9713	86,5351
GelMA+HAMA (2)	0,0012		94,0989	

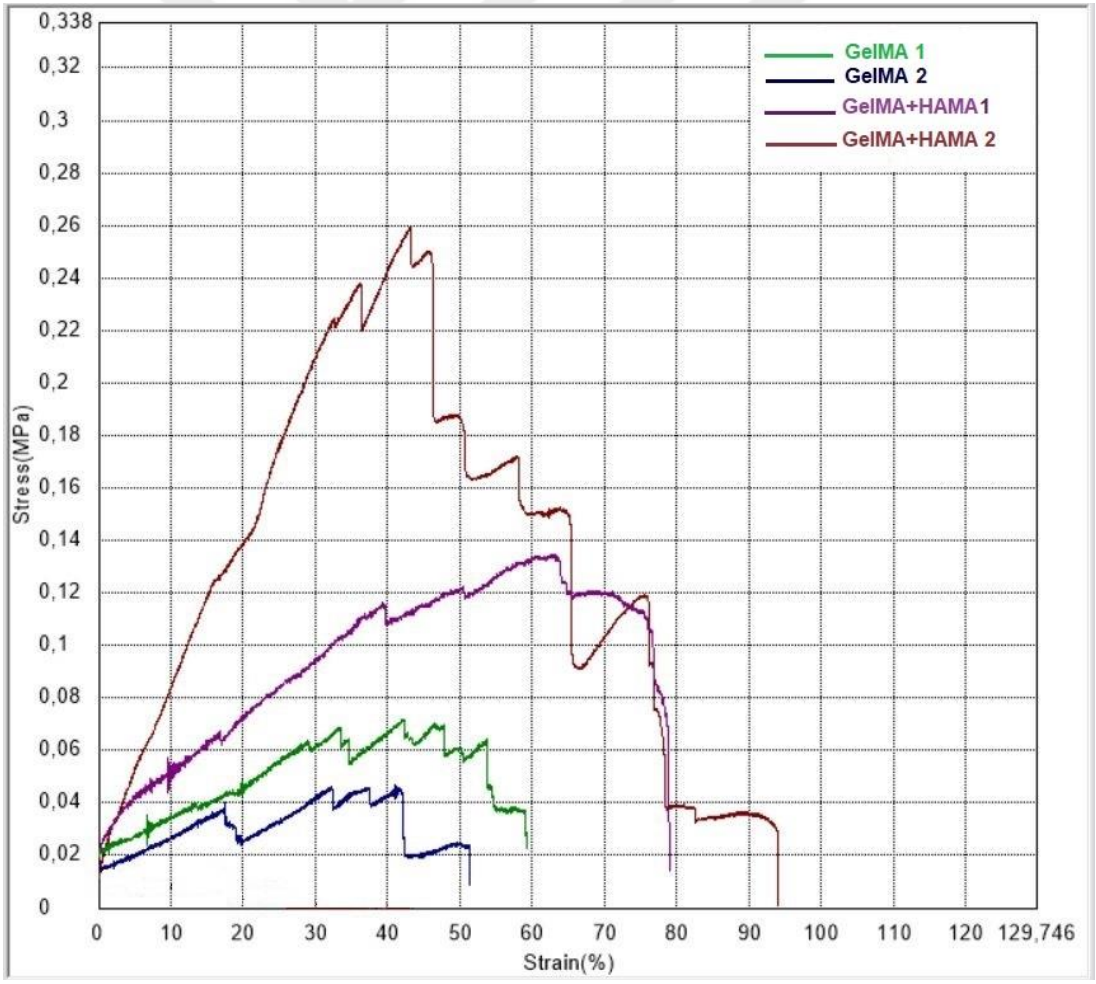


Figure 3.12. The tensile strength values for GelMA and GelMA-HAMA 3D structures.

3.5. Swelling Ratio and Degredation Test for 3D Soft Tissue Engineered Structures

3D bioprinted structures contain water close to one hundred percent, and have the ability to protect it in their 3D structures. Swelling capacity of hydrogels is an sign of the degree of hydrophilicity and is affected by scaffolds pore size [14].

In this study, the swelling behavior of GelMA and GelMA-HAMA structures was found as shown in *Figure 3.12 and 13*. According to the results, the adding 1% (w/v) HAMA in of GelMA solutions was caused the decreasing in mass swelling rate. These results are waited to occur, because the increased polymer density results in higher crosslinking density, as previously reported in the literature [14, 22]. Thus, the resulting structures have smaller pore dimensions and cause less swelling rate in comparison with lower polymer density.

Table 8. Swelling ratio test results for GelMA and GelMA+HAMA 3D structures

3D structures	Swelling ratio (%)	Average
GelMA	580,65	633,58
	690,05	
	629,45	
GelMA + HAMA	620,26	602,50
	589,97	
	597,28	

Table 9. Degredation test results for GelMA and GelMA+HAMA 3D structures

3D structures	(%) Mass Loss for 1st hour	(%) Mass Loss for 4th hour	(%) Mass Loss for 6th hour	(%) Mass Loss for 24th hour
GelMA	357,70	477,80	478,90	40,40
	380,60	393,50	468,70	22,60
	324,90	204,90	93,90	83,60
Average	354,40	358,73	347,17	48,87
GelMA + HAMA	287,80	247,90	245,70	73,30
	323,80	377,00	424,90	34,90
	329,80	352,90	348,20	67,50
Average	313,8	325,93	339,60	58,57

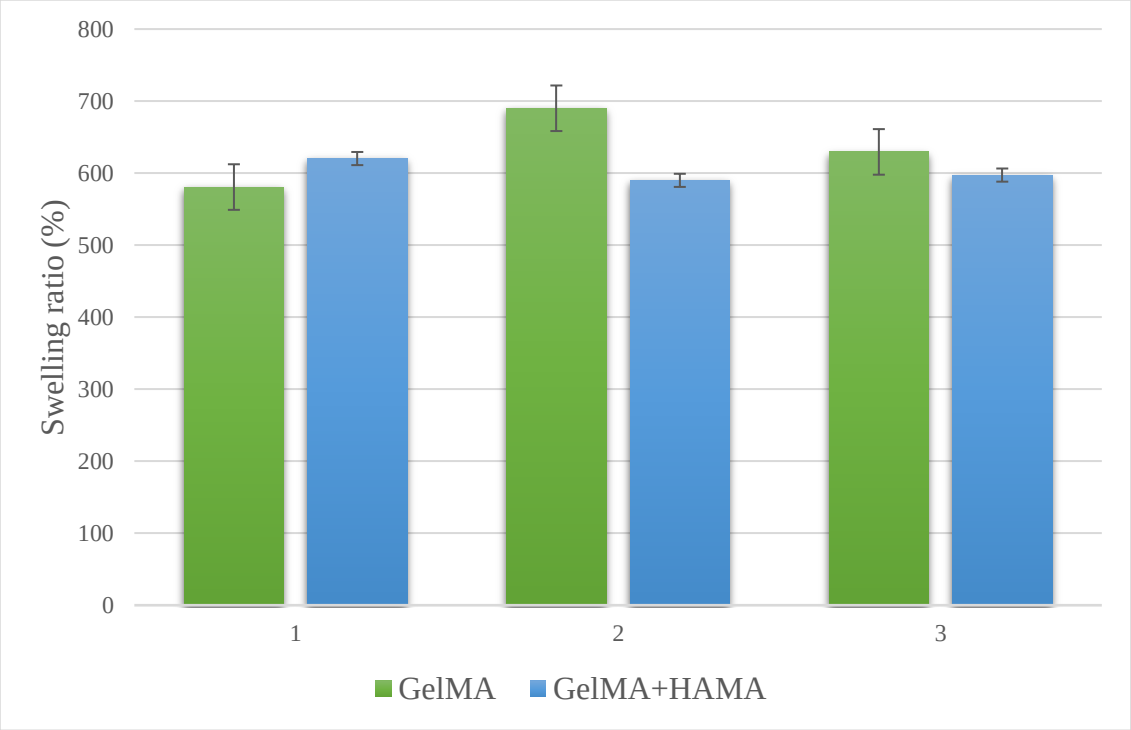


Figure 3.13. Swelling rate of GelMA and GelMA+HAMA 3D constructions

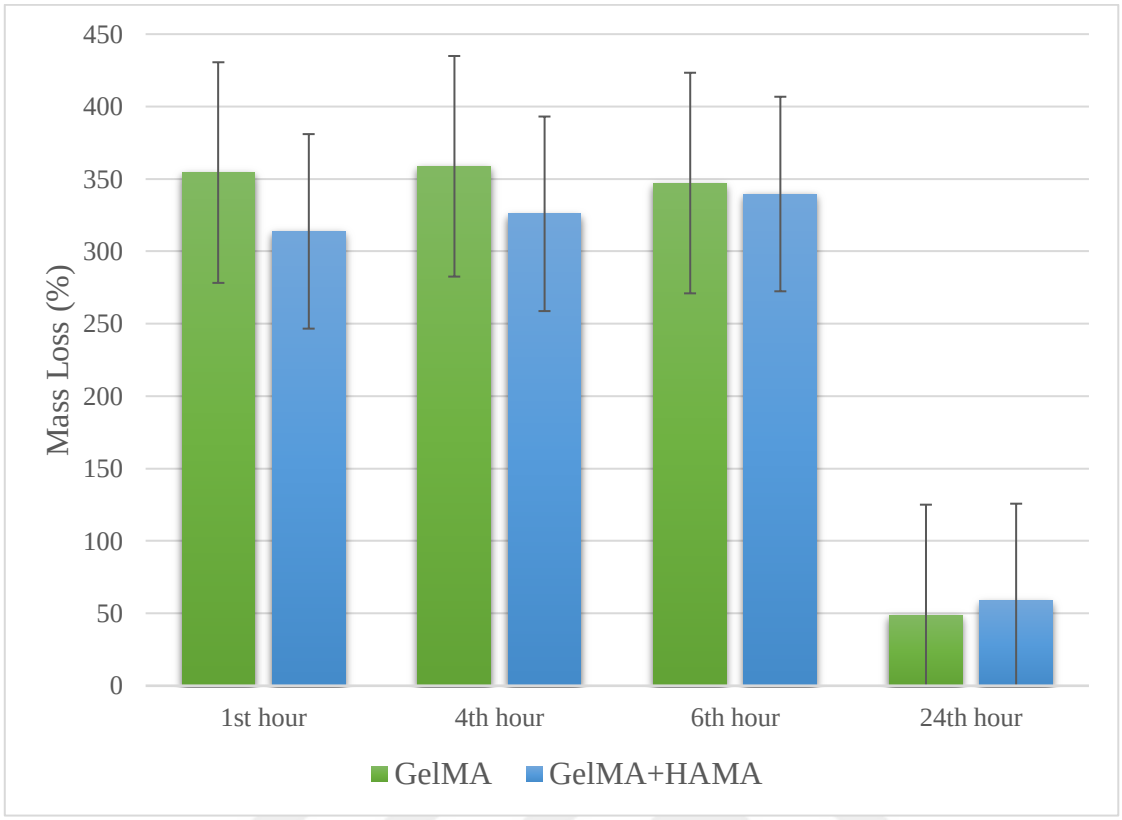


Figure 3.14. Degradation of GelMA and GelMA+HAMA 3D constructions

3.6. *In vitro* Study for 3D reconstructions

Tissue engineering scaffolds and their degradation products should not be toxic to the cells. Therefore, in vitro biological properties of tissue culture plate and 3D printed GelMA and GelMA+HAMA scaffolds were examined by cell viability and cell-scaffold interaction. The result did not show that the proliferation rate of human fibroblast cells significantly increased in all 3D printed scaffolds compared to tissue culture plate after 1 day of incubation. While the cells on GelMA scaffolds showed 31,5 % viability for 1st day incubation, it was 31,6% for the cells on GelMA+HAMA structures. While the cells on GelMA scaffolds showed 63,7 % viability for 4st day incubation, it was 43,8% for the cells on GelMA+HAMA structures. While the cells on GelMA scaffolds showed 59,9 % viability for 7th day incubation, it was 56,5% for the cells on GelMA+HAMA structures.

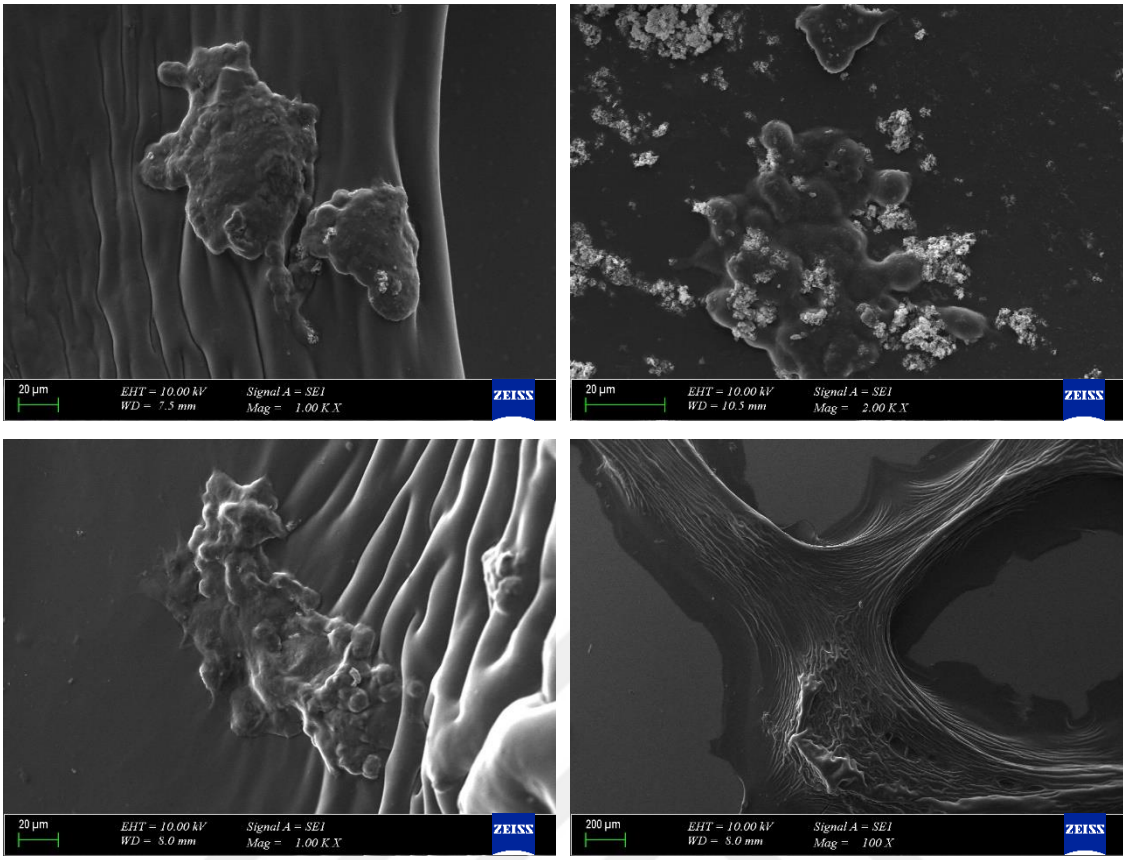


Figure 3.15. SEM images of human fibroblast cells on GelMA scaffolds.

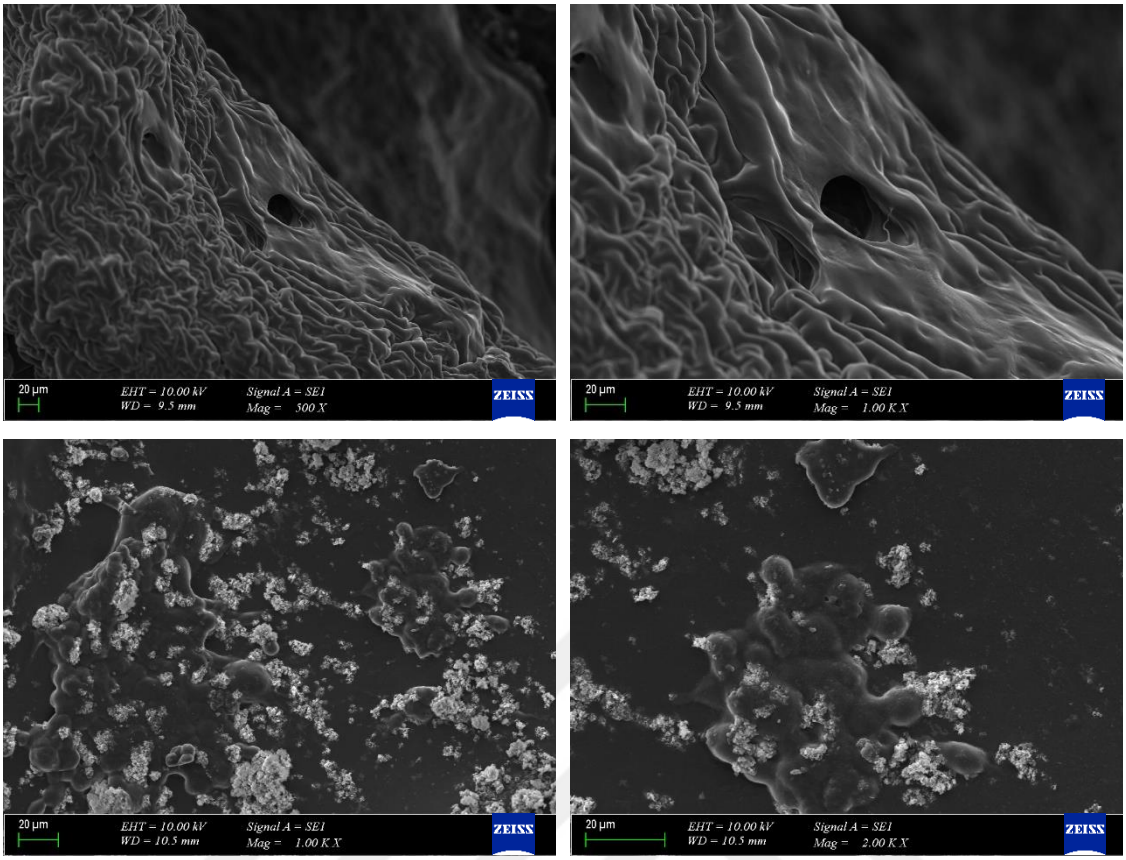


Figure 3.16. SEM images of human fibroblast cells on GelMA+HAMA scaffolds.

The results clearly showed that there was no significant difference between the cell morphology of fibroblast cells on the both GelMA and GelMA+HAMA scaffolds. But the viability was not be expected values for cells on both scaffolds. When the results have been researched, the reason has been based on to be toxic of the methacrylic anhydride solution. On the other hand, it could be caused from durationof dialysis. In this study, duration of dialysis and lyophilization were just for three days. To remove the methacrylic anhydride, the period could be extended.

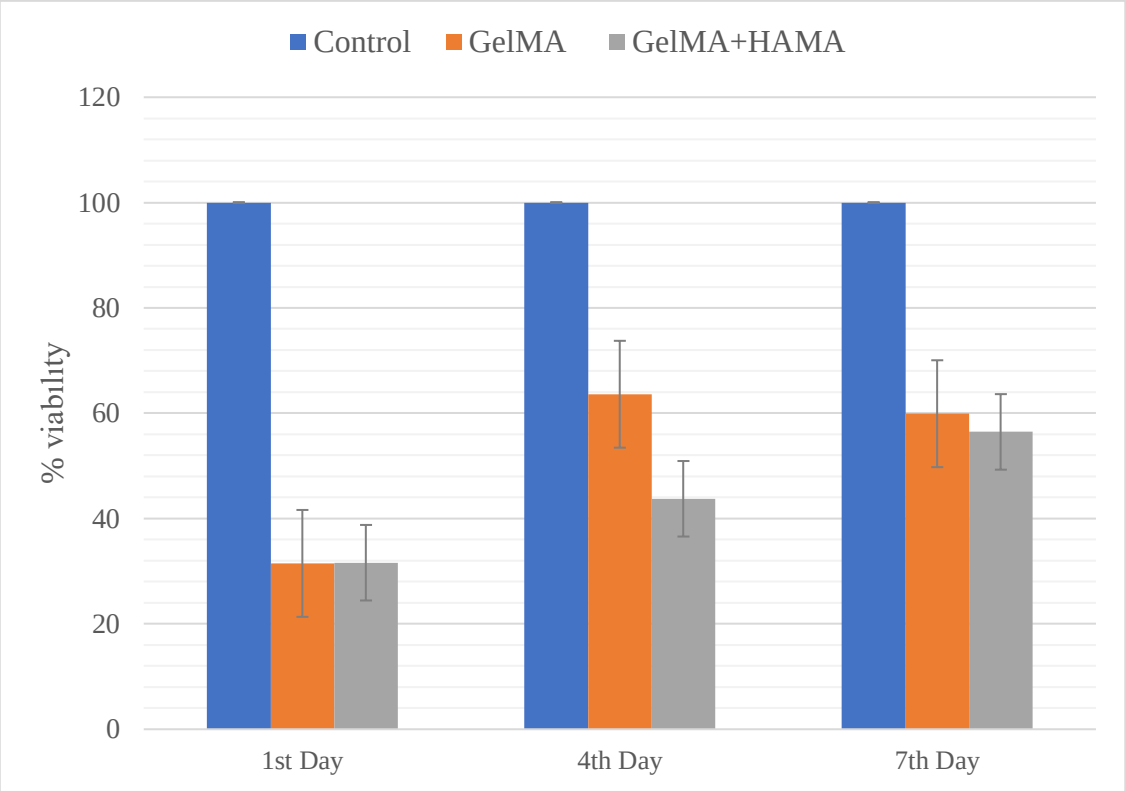


Figure 3.16. Cell viability analysis of GelMA and GelMA+HAMA scaffolds.

4. CONCLUSIONS

In this thesis, musculoskeletal and skin tissue grafts, hernia repair, genitourinary reconstruction, urinary incontinence, dural repair, ligament reconstruction, vascular conduits etc. design and production of body-compatible material similar to tissue; to provide the production of polymeric texture structure in desired dimensions and shapes with the 3D bioprinter method to be made within the thesis. Besides, production of tissue produced in a porous structure to promote cell studies such as cell growth, differentiation, proliferation; synthesis of GelMA, which is a highly biocompatible material in the production of structures similar to the texture in living organisms, and the characterization of these structures is aimed with this thesis.

Methacrylated gelatin and hyaluronic acid were successfully used to produce hydrogels with UV light crosslinking. We have identified the material features of the final hydrogels and pressed them in 3D. The properties of 3D structures were characterized as physically and biologically. Likewise, hydrogels which were generated by the adding of HAMA in GelMA, showed a little less tensile strength but more elongation at break in terms of mechanical properties compared to their properties of structures. Thermal properties shows us there is not considerable differences between them. Their cell interaction biologically was analysed with fibroblast cells. The capability to definetely control physical and biological features of 3D constructs can facilitate off-the-shelf tissue production safely. On account of their plenty in the natural extracellular matrix, hyaluronic acid, and gelatin constructions have perfect potential to be utilized for in different biomedical studies, varying between cell transplantation to tissue engineering.

With those information; tissue applications and regenerative medicine form the basis of engineering and life sciences to improve, repair and improve skin tissue damage and regeneration. Recently, it has been aimed to produce 3D and biocompatible soft tissue engineered structures with 3D bioprinting technology which has a common academic usage.

In addition to the biocompatibility of polymeric soft tissue structures obtained with biodevers in the literature, it will be possible to work with multiple cells.

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CURRICULUM VITAE

Name-Surname : Hatice Merve CAN

E-Mail : can.haticemerve@gmail.com

Languages : Turkish (native), English (IELTS 6.0)

Education : **Bachelor Degree** – Fırat University, Engineering Faculty, Bioengineering (2009 – 2013)

Master Degree[1] **–** Marmara University, Institute of Health Sciences, Pharmaceutical Biotechnology (2015 – 2019)

Master Degree [2] **–** Marmara University, Instıtute For Graduate Studıes In Pure And Applıed Scıences, Bioengineering (2015 – 2019)

Experiences : **Notice Belgelendirme Muayene ve Denetim Hizmetleri Ltd. Şti –** Auditor & Technical Expert and Plannning Responsible (July 2016 – Currently)

Andromed Medikal A.Ş - Project Assistant (July 2013 – June 2016)

Marmara University / Centre for Nanotechnology and Biomaterial Application and Research – Researcher (March 2014 – June 2015)

Ministry of Food Agriculture and Livestock – Intern (July 2012 – September 2012)

Yeditepe University – Brain Research Laboratory and Experimental Animal Research Center – Intern (July 2011 – September 2011)

Papers Presented in National Scientific Meetings and Published in Proceedings

Article:

- Komur B., Lohse T., Can H.M., Khaliilova G., Gecimli Z.N., Aydogdu M.O., Kalkndelen C., Stan G.E., Sahin Y.M., Sengıl A.Z., Suleymanoglu M., Kuruca

S.E., Oktar F.N., Salman S., Ekren N., Ficcai A., Gunduz O. (2016) Fabrication of naturel pumicee/ hydroxyapatite composite for biomedical engineeriing, BioMed Eng OnLine, 15:81.

Presentation:

- **H. Merve Can;** The investigation of the effects of Chitosan solutions in 2D and 3D skin cell culture models, EMBO/EMBL Symposium: Organoids: Modelling organ development and disease in 3D culture (12-15, Oct.2016), Heidelberg / Germany.
- **H. Merve Can;** 3D Printing of Soft Tissue Engineered Constructs for Skin Reconsruction, Bioceramics – 31 and Annual Meeting of the International Sciety for Ceramics in Medicine that held in New Orleans, LA, USA from November 14 – 17, 2019.