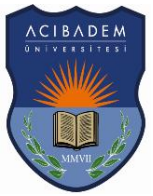


REPUBLIC OF TURKIYE

ACIBADEM MEHMET ALİ AYDINLAR UNIVERSITY

INSTITUTE OF NATURAL AND APPLIED SCIENCES

PEPTIDE ENCAPSULATED AND 3D PRINTABLE HYDROGELS AS POLYMERIC BIOMATERIAL INKS AND THEIR IN VITRO EVALUATIONS

EMİNE DURUKAN

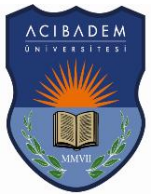
MASTER THESIS

DEPARTMENT OF BIOMEDICAL ENGINEERING

SUPERVISOR

Asst. Prof. Dr. Özgül Gök Özatay

İSTANBUL - 2024



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Tez Onay Sayfası

DECLARATION

I declare that this thesis has been composed by myself and it has not been submitted in any previous application for any other degree. Except for collaborative contributions, the experimental work and the data analysis were entirely done by my own. References have been used for supporting literature.

Emine Durukan

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ACKNOWLEDGMENT

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Finally, I dedicate this research to Palestinian children, sisters and brothers who have been oppressed since October 7, 2023 and ignored by the world. May Allah help them and give them a new future better than their expects.

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LIST OF ABBREVIATIONS

Al	Alginate
Al-MA	Alginate-Methacrylate
Au	Chemical Symbol of Gold
Ag	Chemical Symbol of Silver
Acn	Acetonitrile
BM	Bone Marrow
β	Beta
β-TCP	Beta Tricalcium Phosphate
$Ca_{10}(PO_4)_6(OH)_2$	Hydroxyapatite
Chi-MA	Chitosan-Methacrylate
CCK-8	Cell Counting Kit-8
CB	Carbonate Buffer
Chi	Chitosan
DMSO	Dimethyl Sulfoxide
DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethylformamide
DIC	Diisopropylcarbodiimide
ddH_2O	Double distilled water
ES	Embryonic Stem
ECM	Extracellular Matrix
FT-IR	Fourier Transform-Near Infrared Spectroscopy

FGF	Fibroblast growth factor
FBS	Fetal bovine serum
GFs	Growth Factors
Gel-MA	Gelatin-Methacrylate
Gel	Gelatin
HA	Hyaluronic acid
Ha-MA	Hyaluronic Acid-Methacrylate
HPLC	High Pressure Liquid Chromatography
hFOB 1.19	Human fetal osteoblast cell line
IGF	Insulin-like growth factor
KRSR	Lysine-Arginine-Serine-Arginine
L929	L Strain Clone 929
LVR	Linear viscoelastic region
MA	Methacrylic Anhydride
MSC	Mesenchymal stem cell
MW	Molecular Weigh
MHz	Megahertz
NGF	Nerve growth factor
PEG	Poly(ethylene glycol)
PCL	Poly (caprolactone)
PLGA	Poly Lactic-co-Glycolic Acid
PEGdiMA	Poly(ethylene glycol)di Methacrylate
PTFE	Polytetrafluoroethylene

PLA	Poly(lactic acid)
PBS	Phosphate Buffer Saline
PEGDA	Poly(ethylene glycol) Diacrylate
PEGMEMA	Poly(ethylene glycol) monomethyl ether methacrylate
RGD	Arginine-Glycine-Aspartate
SEM	Scanning Electron Microscopy
Ti-Co	Titanium and Cobalt
TUSEB	Healthy Institutes of Türkiye
TIS	Triisopropylsilane
TFA	Trifluoroacetic acid
UC-MSC	Umbilical Cord Mesenchymal Stem Cells
UV-Vis	UltraViole-Visible
VEGF	Vascular endothelial growth factor
3D Printing	3 Dimensional Printing

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SUMMARY

Peptide Encapsulated and 3D Printable Hydrogels as Polymeric Biomaterial Inks and Their in vitro Evaluations

In this project, natural polymers with biocompatible and biomimetic scaffolds were used to give specific shapes using a 3D bioprinter, especially for application in bone tissue engineering. These natural polymers were modified with methacrylic anhydride so that they could be crosslinked under UV light (365 nm) during the 3D printing process. In addition, the methacrylated polymers were mixed with a synthetic biodegradable polymer PEGdiMA (Poly(ethylene glycol)di Methacrylate) to improve the mechanical strength of the hydrogels. These hydrogels were decorated with a peptide, specific to bone cell adhesion. This peptide includes 4 aminoacid KRSR motifs in its structure and was synthesized via a solid phase peptide synthesis method. By encapsulating the KRSR (osteoblast adhesive peptide) in the polymer, it was possible to immobilize the peptide into the final hydrogel. The obtained 3D bioprintable hydrogels were characterized for their swelling capacity in water, and chemical integrity via FT-IR Spectroscopy, together with its morphological Scanning Electron Microscopy and mechanical (rheometer) properties. Regarding the in vitro analysis, the cytotoxic effect of prepared hydrogels has been investigated against mouse fibroblasts (L929 cell line) via CCK-8 assay and it was demonstrated that prepared hydrogels of various combinations of both natural and synthetic polymers provided more than 70 % cell viability, after which the cell adhesion assay was performed against hFOB 1.19 human fetal osteoblast cell line in a time-dependent manner via live/dead fluorescent imaging-based assay and mineralization rate was determined by Alizarin Red S staining.

Keywords: Hydrogels, Natural Polymers, 3D Bioprinting, Peptide encapsulated Hydrogels, KRSR

ÖZET

3B Basılabilir Hidrojeller için Peptit Kapsüllenmiş ve Hibrit Polimerik Biyomalzeme Mürekkebi Geliştirilmesi ve in vitro Değerlendirilmeleri

Önerilen tez kapsamında, biyouyumlu ve biyomimetik yapı iskelelerine sahip doğal polimerler, özellikle kemik doku mühendisliğinde kullanılmak üzere, 3B biyoyazıcı kullanılarak belirli bir şekil vermek için kullanılmıştır. Bu doğal polimerler, 3B baskı işlemi sırasında UV ışığı (365 nm) altında çapraz bağlanabilmeleri için metakrilik anhidrit ile modifiye edilmiştir. Ek olarak, hidrojellerin mekanik mukavemetini arttırmak için metakrilatlı polimerlere sentetik ve biyobozunur bir polimer olan PEGdiMA (Poli(etilen glikol)di Metakrilat) eklenmiştir. Bu hidrojeller, kemik hücresi yapışmasına özel bir peptidin kapsüllenmesiyle elde edilmiştir. Katı faz peptid sentezi yöntemiyle sentezlenen bu peptid, yapısında 4 aminoasit KRSR motifi içerir. KRSR'nin (osteoblast yapışkan peptid) polimere kapsüllenmesiyle, peptidin hidrojellere immobilize edilmesi mümkün olmuştur. Elde edilen 3B basılabilir hidrojeller, morfolojik Taramalı Elektron Mikroskobu ve mekanik (reometre) özelliklerinin yanı sıra, sudaki şişme kapasiteleri, FT-IR Spektroskopisi yoluyla kimyasal bütünlükleri açısından karakterize edilmiştir. *In-vitro* analize ilişkin olarak, hazırlanan hidrojellerin fare fibroblastlarına (L929 hücre hattı) karşı sitotoksik etkisi CCK-8 kiti ile araştırılarak hem doğal hem de sentetik polimerlerin çeşitli kombinasyonlarından hazırlanan hidrojellerin %70'ten fazla hücre canlılığı sağladığı gösterilmiştir. Daha sonra insan fetal osteoblast (hFOB 1.19 hücre hattı) hücreleri üzerinde live-dead floresan görüntüleme boyası kullanılarak hücre adezyon testi yapılmış ve Alizarin Red S boyamasıyla mineralizasyon oranı tespit edilmiştir.

Anahtar Kelimeler: Hidrojeller, Doğal Polimerler, 3D Biyobaskı, Peptit Kapsüllenmiş Hidrojeller, KRSR

1. BACKGROUND AND AIM OF THE STUDY

In this project, we aimed to produce biocompatible and biomimetic hydrogel-based scaffolds that can be given the desired shape by using a 3D bioprinter, especially for application in bone tissue engineering. The properties of natural biopolymers that we used in tissue scaffolds such as highly biocompatible, biodegradable, low-budget, time-saving, and easily accessible have made natural biopolymers frequently used in biomedical applications. Based on this, it was thought that functionalization with a synthetic polymer polyethylene glycol dimethacrylate (PEGdiMA) would support the low mechanical properties of these biopolymers to be functionalized with methacrylate groups. On the other hand, to increase cell adhesion, encapsulating scaffolds with KRSR peptide which is specific to osteoblast cells was a useful strategy in the production of bone tissue-specific scaffolds. The potential of hybrid polymer blends to be produced using this strategy is high, both to be presented as a commercial product for research purposes and to be an effective medical treatment tool by printing in patient-specific forms.

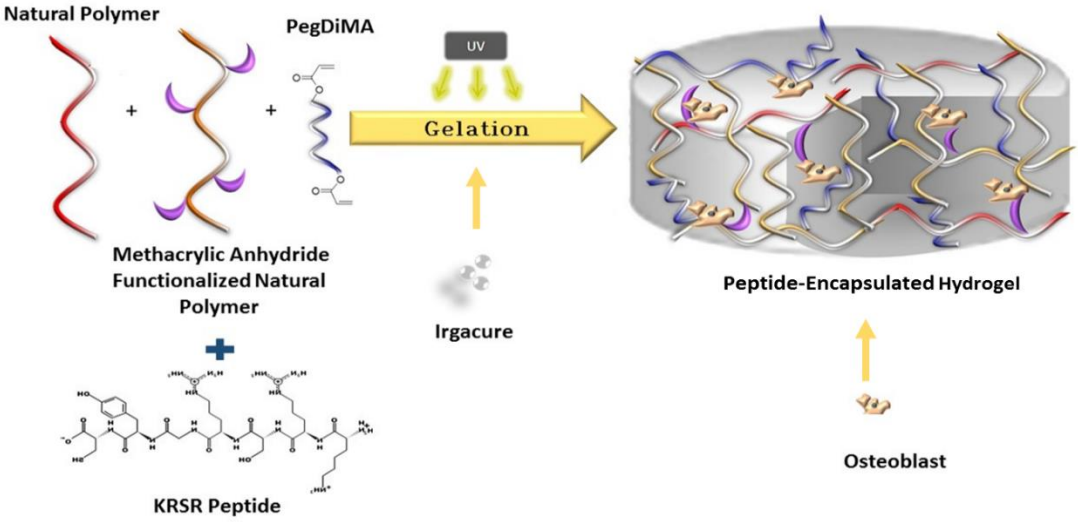

Figure 1.1 Preparation of 3D printable scaffold systems.

2. INTRODUCTION

2.1. Tissue Engineering

When tissues or organs are lost due to disease, congenital anomaly, cancer or trauma that conventional pharmaceutical treatments are no longer applicable, tissue or organ transplantations are the first choice for the reconstruction of destroyed tissues or organs. However, today these surgical treatments face some difficulties. Artificial organs have been developed in recent years with significant advances in biomedical engineering, but there is still a need for better biocompatibility and biofunctionality. Although immuno-suppressive therapy has recently become much more advanced, problems with current organ transplantation include insufficiency of donated organs and immune rejection (1). On the other hand, the increase in the number of people waiting for organ transplantation and the shortage in the number of organ donors has made it necessary to develop new methods to restore the functions of damaged tissues and organs (2). According to the data of the Turkish Ministry of Health, a total of 26,894 patients are waiting in line for transplantation in our country in 2023. In this framework, the goal of tissue engineering is to diminish the critical deficiency in donor organs by using *in-vitro* production of functional biological structures.

The field of tissue engineering is highly interdisciplinary field that puts together experts such as biomedical engineering, clinical medicine, mechanical engineering, material science, genetics and related sciences. The field stands for the use of 3D scaffolds to provide a suitable environment for cells, tissues and also growth factors that stimulate the cells for differentiation and proliferation.

Lastly, cells are needed as cell-loaded scaffolds to culture *in-vitro* to synthesize tissues that can be implanted in damaged and injured areas in *in-vivo* (3). These combinations can be summarized as tissue engineering consisting of biomaterials, growth factors and cells as shown in Figure 2.1.

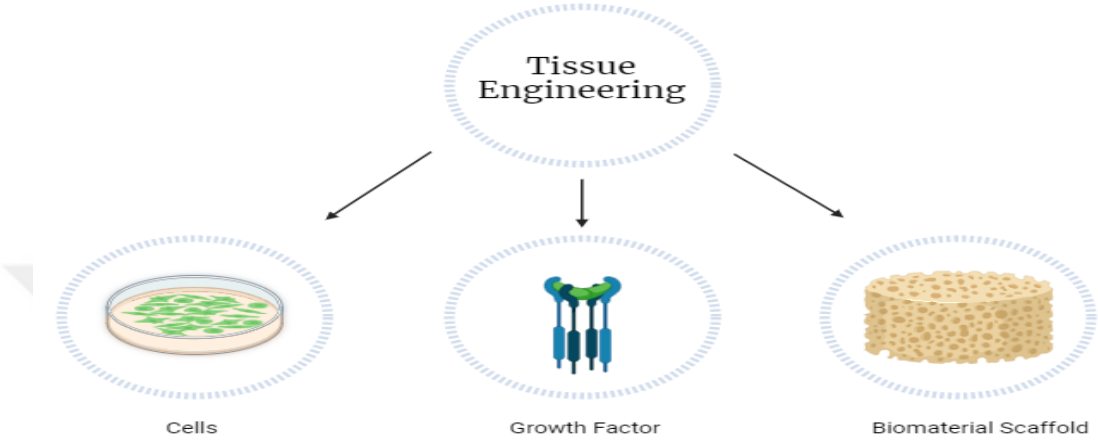

Figure 2.1 Components of Tissue Engineering; Biomaterial Scaffold, Growth Factor and Cells.

Growth Factors (GFs) are soluble and crucial molecules for tissue regeneration, healing, and repair and stimulate cell migration, proliferation, and differentiation. Therefore, it's promising for regenerative medicine applications (4).

There are different types of Growth factors that are used for different aims as shown in Table 2.1 (5).

Table 2.1 Different GFs for different aims (6).

Growth factors	Clinical application
Vascular endothelial growth factor (VEGF)	Regeneration of epithelial tissue
Fibroblast growth factor (FGF)	Angiogenesis, bone repair, treatment of diabetes, nerve growth
Insulin-like growth factor (IGF)	Cell proliferation, differentiation, development and growth
Nerve growth factor (NGF)	Treatment of neurodegenerative diseases

Fabrication of engineered tissue in an artificial environment requires the use of cells to populate matrices and produce matrices similar to native tissue. The key point to be successful here is to use primary cells taken from the patient and combined with the scaffolds to produce implants. However, this strategy has limitations because of the potential for cells to be diseased. In this framework, using stem cells has been remarked in tissue engineering. including mesenchymal stem cells (BM-MSCs), bone marrow, embryonic stem (ES) cells, umbilical cord mesenchymal stem cells (UC-MSCs) (7).

2.1.1 Bone Tissue Engineering

Bone tissue is composed of two different components; cortical and cancellous bone. The inner part which is cancellous bone is spongy and has 50-60 % porosity however outer part which is cortical bone is denser with 10 % porosity. Both of these bone structures endure differentiation, maturation which are controlled through osteocyte, osteoclast, and osteoblast cells. Osteoblasts are responsible for new bone formation. Osteoclasts are responsible for the absorption of old bone.

Osteocytes release hydrolysis enzymes (8). These cells are also responsible for maintaining the health of bone (9).

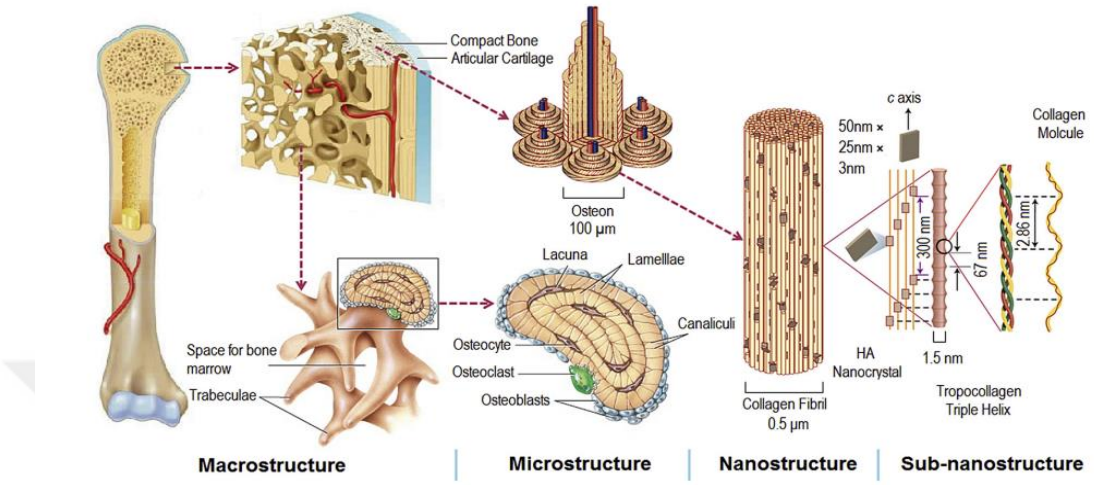

Figure 2.2 Hierarchical Structure of Bone (10).

The natural formation of bone and its hierarchical structure during fracture healing and embryonic development inspire bone tissue engineering. Bone tissue consists of organic material (especially collagen type I), inorganic materials (especially calcium phosphate) and different types of cells. The Extracellular Matrix (ECM) which is the main aim for tissue engineering consists of crosslinked fibrils of collagen. The outer part has dense compact bone while the inner part consists of cancellous bone which is called trabeculae. The researchers mainly and commonly aim to mimic the physical structures of bone such as minerals, protein, and cellular components of bone to maintain the growth of bone tissue. Additionality, non-human organisms such as plant or animal tissue structures inspire the biomaterials of bone tissue engineering (11).

Although bones can renew themselves, they can sometimes fail especially when large-scale bone damage can't heal by the body. In this case, some of methods are used to restore bone function such as autograft (taken from same body), allograft (taken from another body), xenograft (taken from an animal), and tissue engineering which needs biomaterial scaffolds that are used to overcome these problems and it aims to promote new tissue formation, cell signaling, regeneration and signaling inside of the scaffold (12).

2.2 Biomaterials for Scaffold Fabrication

The use of biomaterial scaffolds in tissue engineering to mimic extracellular matrix (ECM) and provide cell-mediated regeneration which are expressed as 3D (three dimensional) porous solid biomaterials to perform some functionalities such as; cell adhesion, cell-material interactions, promote cell proliferation and differentiation, allow cell survive and transportation of nutrients, gases, growth factors (GFs) (13).

Biomaterials are literally defined as materials used in medical field and in contact with living organisms to perform original replaced organ (14). In ancient times, people used animal or plant-derived materials to heal their wounds or sustain their lives. Later, with the development of industry, people were able to use synthetic biomaterials (metallic and polymeric). Nowadays, different types of biomaterials have been used such as metals, ceramics, natural and synthetic polymers, composites and hydrogels in tissue engineering. The common property of all of them is that they are biocompatible, bioactivity and biodegradability (15).

Biocompatibility which is one of the main requirements of biomaterials is important for cell adhesion, migration and proliferation after implantation and reduces immune response. Bioactivity demonstrates the ability of the interaction of the biomaterials with surrounding tissue to provide cell adhesion, proliferation, migration and differentiation while biodegradation represents allowance to produce the cells their own Extracellular Matrix (ECM) in the biomaterial scaffold thus it prevents inflammation (15).

Ceramics can be natural or synthetic which is an alternative to metallic biomaterials and defined as nitrides, oxides, carbides and sulfides of metals and metalloids. These are important features due to mimicking the natural human or animal body. They have excellent biocompatibility, poor degradability, non-corrosive, good mechanical properties. Zirconia, alumina, titania, Ca/P, and glasses are used in orthopedics, dentistry, implants, calcified tissues, medical sensors and coatings (16). Composite biomaterials are defined as a combination of two or more materials to get a biomaterial to be better than the individual.

The advantages of composites are specific stiffness, biocompatibility and allowing specific design etc. Bioceramic composites such as zirconia, alumina, Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$); Polymers composites such as Polytetrafluoroethylene (PTFE), poly-dicyclopentadiene, polyimide, poly(caprolactone) (17).

Metals are commonly known for load-bearing biomaterials and widely used in orthopedics, dental surgery, and cardiovascular surgery. They vary from screws, and wires to the joint, ankle, knees and hip prostheses etc. Stainless steels, titanium and cobalt (Ti-Co) alloys, tantalum-tungsten alloys mostly use metallic biomaterials. The main advantages of metallic biomaterials are stiffness, and strength while they have low corrosion resistance which can be toxic to the surrounding tissues (18).

Polymers are very useful in biomaterial applications for tissue engineering scaffolds and medical devices. It's also important to select suitable materials as biomaterials according to their chemistry, shape, solubility, molecular weight, lubricity, hydrophobicity-hydrophilicity, surface energy, etc. Polymeric scaffolds are paying attention due to their unique features such as high porosity, biodegradation, mechanical properties and easily modifiable. They are used in bone and cartilage tissue engineering, drug delivery systems and gene therapy. There are different types of polymeric scaffolds such as hydrogel scaffolds, porous scaffolds, microsphere scaffolds and polymer-ceramic scaffolds, etc (19). Different types of biomaterials are used to build scaffolds such as composites, ceramics, metals, natural and synthetic polymeric hydrogels as shown in Table 2.2.

Table 2.2 Different types of Biomaterials (20).

Materials	**Advantages**	**Limitations**	**Applications**
Metals (Au, Ag, Ti and alloys, stainless steel)	Stiffness, strength, tensile strain, resistance.	Easily destroyed by rust Elasticity, Low corrosion resistance.	Bone screws-pins and plates, dental implants, joint replacements etc.
Composites	Resistance to rust, strength, biocompatible, eco-friendly.	Required homogeneity, hard construction.	Bone cement, joint replacements, dental implants, calcified tissues, medical sensors, orthopedics
Ceramics (Zirconia, Alumina, Hydroxyapatite, silica, glass, titanium dioxide, etc).	Eco-friendly, rust resistance, strength.	Hard, not flexible, variable mechanical strength.	Load-bearing implants, biosensors, orthopedic and dental implants.
Polymers (Nylon, polyester silicon, gelatin, chitosan, etc)	Non-toxic for tissues, less load, easy to construct.	Less strength, non-stable shape and degradation.	Contact lenses, heart valves, hip joints, wound patches etc.

2.2.1 Polymeric Hydrogels

Polymeric hydrogels consist of three-dimensional and crosslinked hydrophilic polymers that uptake water and body fluid.

Hydrogels have excellence properties since they promote the formation of the ECM, provide cell migration and proliferation, release of the growth factor, drugs, and diffusion of nutrient substances that support cell proliferation (21) as shown in Figure 2.2.

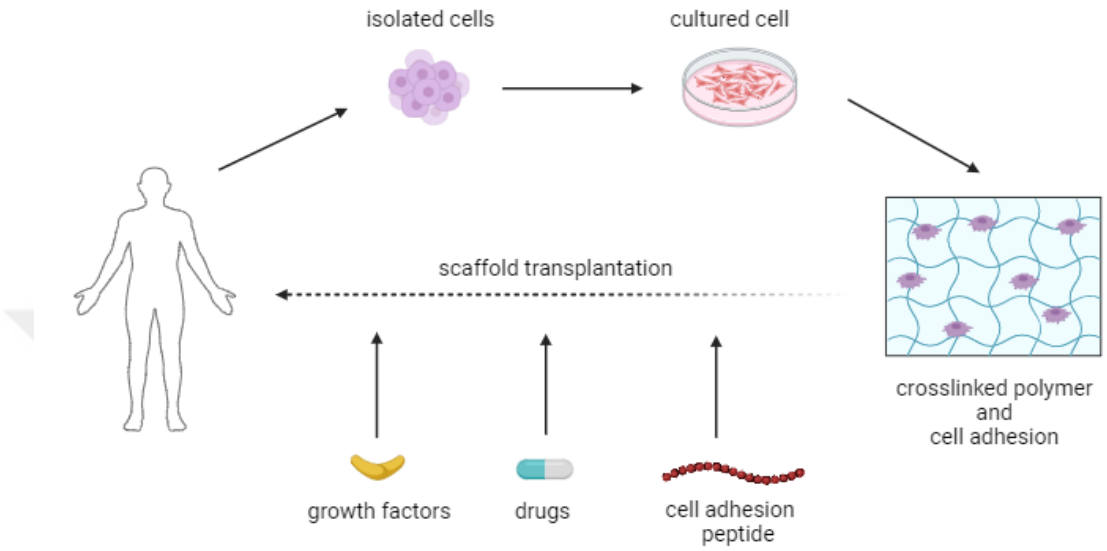

Figure 2.3 Scaffold transplantation facilitates the release of growth factors, drugs and provides cell adhesion (adapted from (21)).

Thus, hydrogels can show excellent properties in the body. Additionally, they are very preferable because of time and cost saving, reproducible, more biocompatible, easily modifiable, and adjustable mechanical properties.

Natural and synthetic polymers can be used for hydrogel synthesis. The main natural polymers are gelatin, alginate, hyaluronic acid, chitosan and fibrin which can mimic natural tissues. However mechanical strength is poor so need to composite with synthetic polymers which are (PEG) poly(ethylene glycol), (PCL) Poly (caprolactone), (PLGA) Poly Lactic-co-Glycolic Acid, (PEGdiMA) Polyethylene glycol dimethacrylate (22), (23).

The polymers mentioned above can be modified with different molecules, and peptides to improve their cell adhesion, tissue development and regeneration. For instance, gelatin has RGD (arginine-glycine-aspartate) peptide which increases cell adhesion to the gelatin-based hydrogels (24). Additionally, KRSR (Lysine-ArginineSerine-Arginine) peptide which is specific to the osteoblast increases osteoblast cell adhesion (25). There are various studies on this subject in the literature. Nelson and his colleagues proved the effect of the KRSR peptide on cell selectivity. Using the KRSR peptide and the bone cell adhesion-inhibiting KSRR peptide as a negative control, they found that human osteoblast cells bound more to KRSR-modified hydroxyapatite than to the KSRR-modified one, and also that the cells spread, migrated, and proliferated more as a result of this functionalization (26). In another study, Hassenbein et al. found that the most common RGD (Arg-Gly-Asp), which is a cell adhesive peptide used for many cells and found naturally in gelatin-collagen polymers. They found that the peptide was not as effective as KRSR on osteoblast cells (27).

2.2.1.1 Natural and Synthetic Polymers

Hyaluronic Acid

Hyaluronic acid and also known as hyaluronan is a non-sulfated and anionic, natural linear polysaccharide that is composed of N-acetyl-D-glucosamine and D-glucuronic acid units linked by β-1,3 and β-1,4 glycosidic bonds. It can be based on animal or bacterial fermentation. Hyaluronic acid (HA) is an important component in synovial fluid and ECM. It's a biocompatible, biodegradable and hydrophilic polymer that promotes cell proliferation and motility. On the other hand, due to its viscous structure, it's very suitable for 3D bioprinting (28). Moreover, it has hydroxyl groups (-OH) on the side chain of the hyaluronic acid which can be used as photo-crosslinkable functional groups.

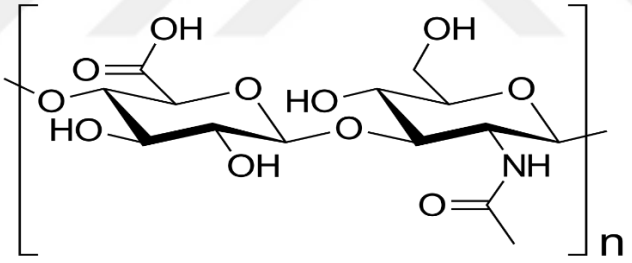

Figure 2.4 Chemical Structure of Hyaluronic acid (29).

Fibrin

Fibrinogen is a natural glycoprotein in 45 nm length and made up of three chains Aα, Bβ, and γ chains. It can be used as injectible biomaterials to fill cavities in the spinal cord (30). Fibrin plays an important role in clotting blood which is highly biocompatible and promotes cell adhesion and proliferation. Since they have poor mechanical properties, they need to be modified with other polymers (31). Moreover, it has amino group ($-NH_2$) groups on the side chain of the fibrin which can be used as photo-crosslinkable functional groups.

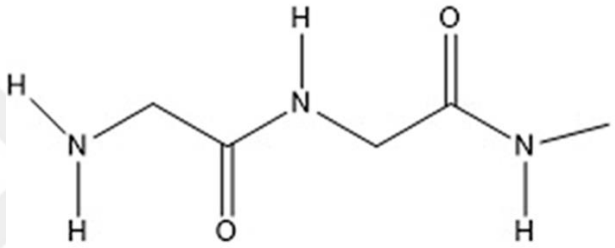

Figure 2.5 Chemical Structure of Fibrin (32).

Alginate

Alginate is a linear, natural and water-soluble polysaccharide produced from bacteria and brown algae. It's approved by Food and Drug Administration (FDA) and is biocompatible, safe. The gelation overcomes in different conditions. At pH under 3, alginate forms self-assembly by forming hydrogen bonds. Moreover, it can form physical crosslinking by binding with $Ca^{2+,}$ Mg^{2+}, Al^{3+} or Ba^{2+}. The alginate chain consists of Guluronic Acid (G unit) and Mannuronic Acid (M unit) (33). On the other hand, it's very preferable for 3D bioprinting due to its viscous structure. Moreover, it has hydroxyl groups (-OH) on the side chain of the alginate which can be used as photo-crosslinkable functional groups.

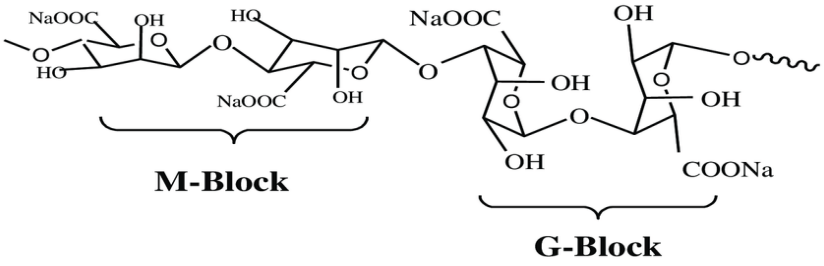

Figure 2.6 Chemical Structure of Alginate (34).

Gelatin

Gelatin is a natural and water-soluble protein generally produced from bone collagen or the skin of animals. It's biodegradable and highly biocompatible by mimicking Extracellular Matrix (ECM) and provides cell adhesion due to Arg-Gly-Asp (RGD) peptide. For this reason, gelatin is frequently used as scaffold in tissue engineering although it has poor mechanical properties.

That's why, gelatin-based bioinks are modified with other polymers such as hyaluronic acid, alginate, PLGA, and PEGdiMA (35). Moreover, it has amino groups ($-NH_2$) and hydroxyl groups (-OH) on the side chain of the gelatin which can be used as photo-crosslinkable functional groups.

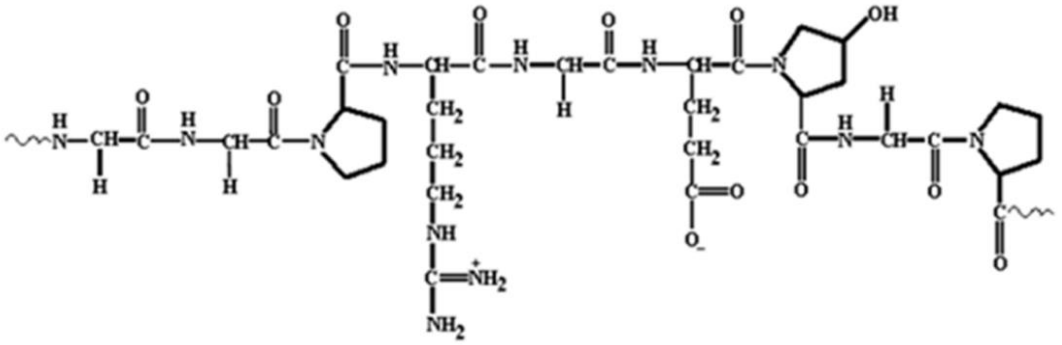

Figure 2.7 Chemical Structure of Gelatin (36).

Chitosan

Chitosan is a cationic amino polysaccharide, non-toxic, biodegradable, natural, biocompatible and hydrophilic polymer which is used in biomaterial research. Its structure is (1–4)-2-amino-2-deoxy-β-D-glucan and obtained from hydrolysis of chitin in the exoskeleton of insects, crabs and fungal cell walls, etc. Since it has a viscous structure, it's very useful for 3D bioprinting as scaffold. Moreover, it has amino groups ($-NH_2$) and hydroxyl groups (-OH) on the side chain of the chitosan which can be used as photo-crosslinkable functional groups (37).

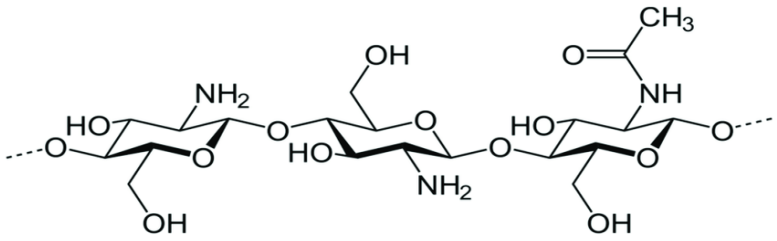

Figure 2.8 Chemical Structure of Chitosan (38).

Poly(ethylene glycol) (PEG)

Poly(ethylene glycol) (PEG) is amphiphilic, synthetic, flexible frequently used in tissue engineering as a scaffold, and 3D cell culture systems due to its cytocompatibility, FDA-approved and drug delivery systems (39). Moreover, it's suitable for 3D bioprinting due to its viscous form and tunable physicochemical properties (40). PEG is liquid between 100-700 molecular weigh (MW) at room temperature, 1000-2000 are solid and PEG with MW > 2000 is a crystalline solid. PEGs are neutral at all pH levels. On the other hand, they are inert in the biological environment with poor cellular uptake and cellular adhesion (41).

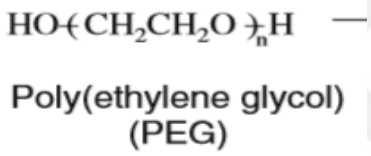

Figure 2.9 Chemical structures of PEG macromer (42).

Polyethylene glycol dimethacrylate (PEGdiMA)

PEG-based hydrogels such as poly(ethylene glycol) diacrylate (PEGDA), polyethylene glycol dimethacrylate (PEGdiMA) are highly used in tissue engineering and drug delivery systems due to their non-toxic, hydrophilic, biocompatible, synthetic, FDA approved and photo-crosslinkable because of methacrylate groups two ends of PEG chain. Since it's bio-inert, needs to be modified with other polymers or molecules to promote cell adhesion (43).

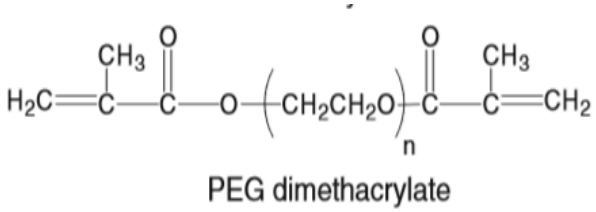

Figure 2.10 Chemical structures of PEG macromer (44).

Poly(caprolactone) (PCL)

Poly(caprolactone) (PCL) is a synthetic, aliphatic, linear, biocompatible, FDA-approved and biodegradable polymer that is used in tissue engineering for scaffold building. Moreover, it has thermal stability which utilizes 3D printability. On the other hand, it's easy to process PCL as desired to use in different tissue defects (45).

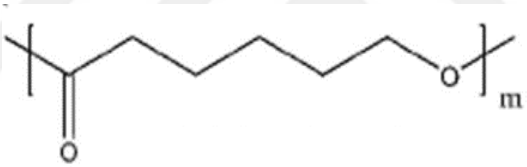

Figure 2.11 Chemical structures of PCL (45).

Poly Lactic-co-Glycolic Acid (PLGA)

Poly (lactic-co-glycolic acid) (PLGA) is a synthetic, amorphous, aliphatic polymer that is used in tissue engineering because of its adjustable biodegradability and good biocompatibility. It's composed of two monomers lactic acid (LA) and glycolic acid (GA). The amount of LA and GA affects the degradation of PLGA. For instance, higher LA causes lower degradation of PLGA. PLGA is used in 3D bioprinting, electrospinning, hydrogels, microspheres, etc (46).

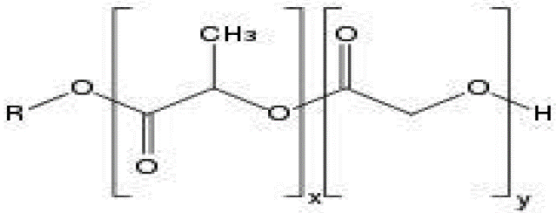

Figure 2.12 Chemical structures of PLGA (47).

2.2.2 Crosslinking of Hydrogels

Hydrogels are macromolecular polymers that form network crosslinked chains of polymer (48). Natural or synthetic polymers are used for 3D networks for bioprinting by using physical, chemical, ionic, light (photo-crosslinking), DNA hybridization, enzymatic etc. (49).

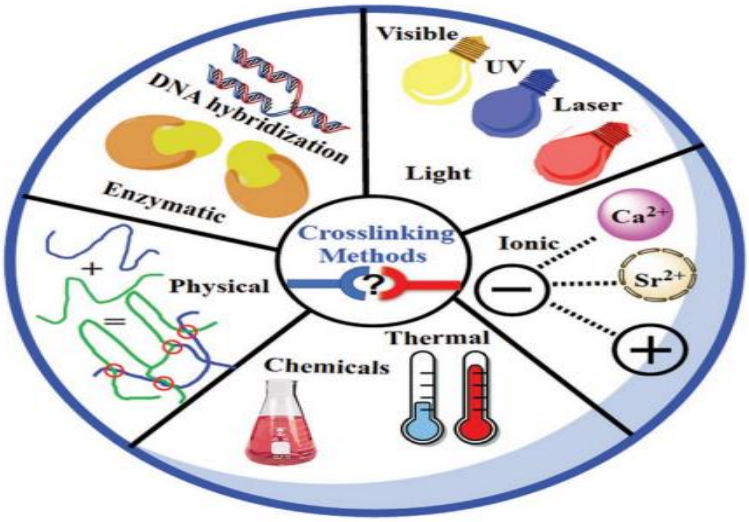

Figure 2.13 Different types of cross-linking methods for polymerization (49).

2.2.2.1 Photo-Crosslinked Hydrogels

Photo-crosslinking is one of the most effective methods to crosslink the polymer chains to form hydrogels covalently for producing stable 3D hydrogel structures in diverse geometries (50). In this method, polymers are modified with functional groups such as acrylates, methacrylates, maleimides that cause photopolymerization in the presence of a photoinitiator (Irgacure 2959, Eosin-Y) under light exposure (51). This crosslinking method facilitates building of 3D structure scaffolds in line with the physiological systems and anatomical architecture of the patient (52). The main idea of photopolymerization is the conversion of liquid starting material induced by irradiation with visible or UV light. Irradiation light can be from 254 nm to 800 nm depending on the light source such as mercury-microwave lamps, xenon lamps (53). UV light is the most used photo source and when compared to visible light UV light has an advantage by using colorless photoinitiator (54). For visible light, thiol-ene reactions and thiol-acrylate photopolymerizations, and acrylates and methacrylates can be used for UV polymerization (55).

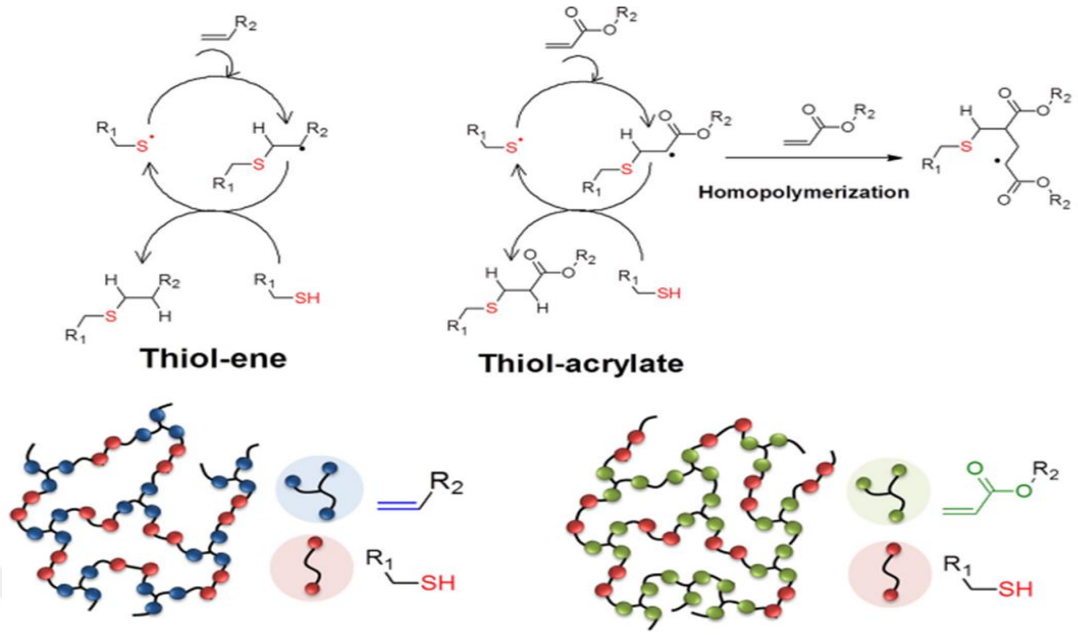

Figure 2.14 Thiol-ene and thiol-acrylate photopolymerization (56).

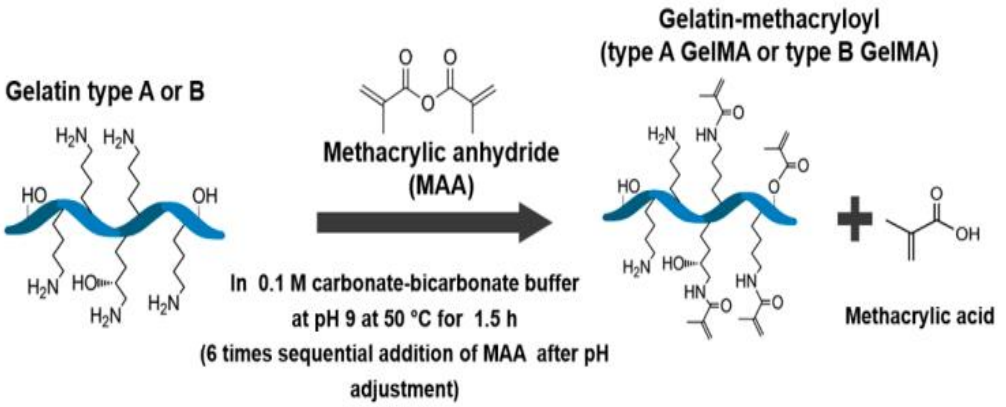

Figure 2.15 Synthesis of Gelatin-Methacrylate (Gel-MA) (57).

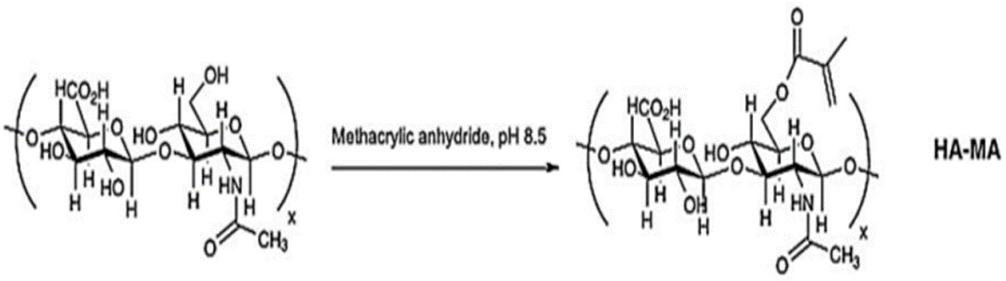

Figure 2.16 Synthesis of Hyaluronic Acid-Methacrylate (Ha-MA) (58).

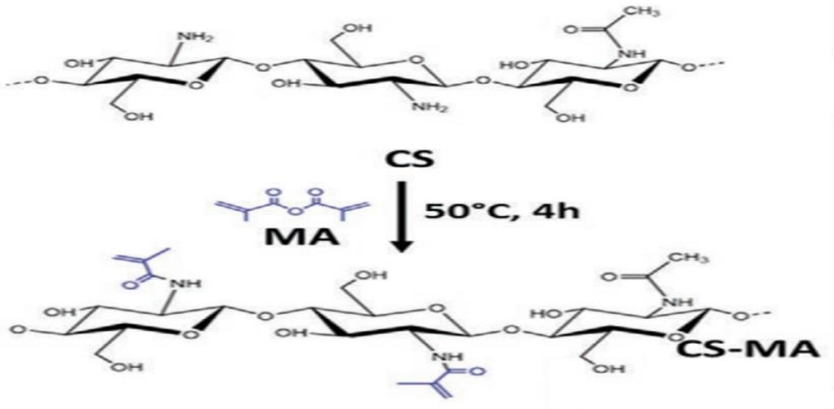

Figure 2.17 Synthesis of Chitosan-Methacrylate (Chi-MA) (59).

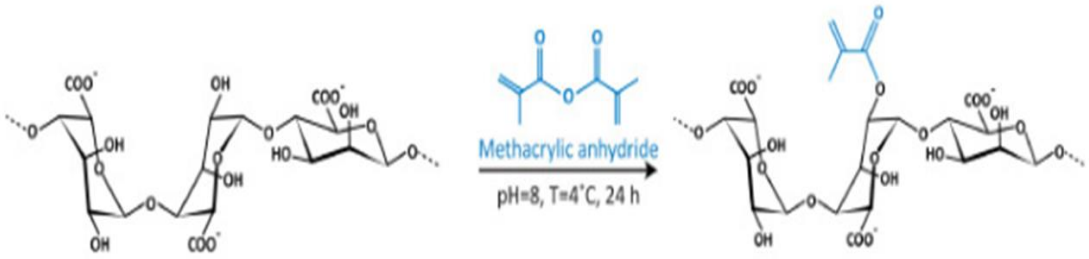

Figure 2.18 Synthesis of Alginate-Methacrylate (Al-MA) (60).

2.2.3 3D Printing of Hydrogels

3D bioprinting has become the most popular technique to build scaffolds and medical devices in tissue engineering. It's because of the specific to patient, low-cost, rapid fabricating. Bioprinting inks are called 'bio-inks' which are printed with cells or 'biomaterial inks' which are printed without cells. In general, printable biomaterials should be biocompatible, biodegradable, cytotoxic for living tissue, and biomimetic the original organ (61).

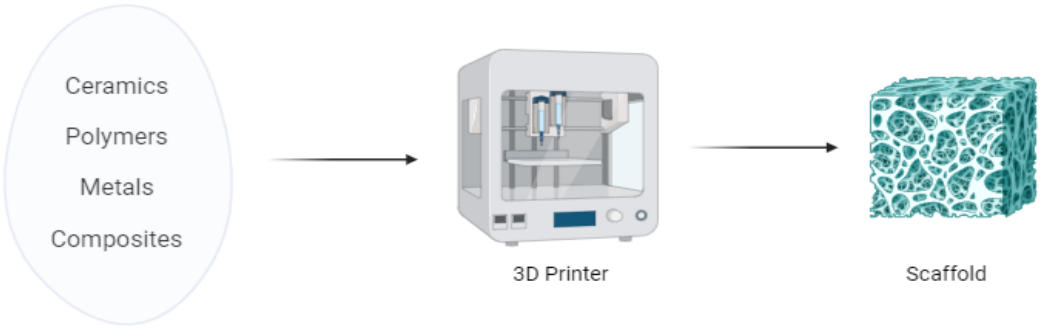

Figure 2.19 Printing schema and biomaterials used for 3D printing (61).

As seen in Figure 2.4, ceramics, polymers, metals, and composites can be used as biomaterial inks. Among the polymers alginate, chitosan, and hyaluronic acid are most used polymers because of their viscosity.

3D printing technologies can vary according to several criteria. These are inkjet bioprinting laser-assisted bioprinting and extrusion bioprinting.

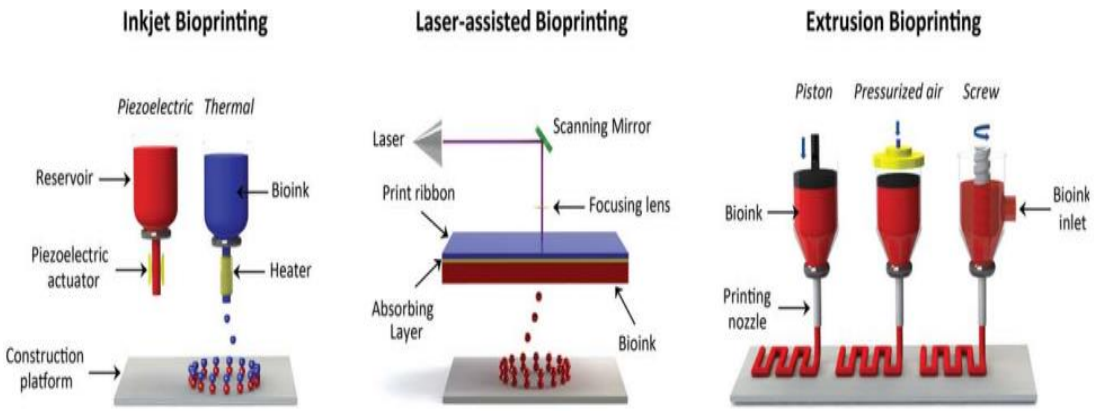

Figure 2.20 Illustration of 3D bioprinting and main components (62).

Inkjet Bioprinting

This is a printing method in which droplets that contain biomaterials and cells are patterned layer by layer on a substrate. This is most used 3D printing method. Basically, the syringe is filled with bioink and piezoelectric actuator that controlled by computer is used to place droplets on the substrate to build a 3D scaffold.

The size of droplet can be adjusted by changing the frequency of pulse of energy. The frequency can be varied from 5 to 300 MHz which changes droplet diameter from 300 to 5 μm. The advantages of inkjet bioprinting are low-cost, high printing capability and speed. However, bioinks that contain cells can aggregate in the syringe and cause the plug syringe's nozzle (63).

Laser-assisted Bioprinting

Laser-assisted 3D printing systems use a laser beam pulsing and two parallel slides which are collector and donor. There is a laser-absorbing metal under the donor slide. As the laser stimules between 65 nJ and 190 μJ energy is absorbed by the metal. This method permits high-resolution in liquid or solid phase. The advantage of this bioprinting method is prevents nozzle clogging and contamination. On the other hand, high cell viability with low mechanical stress during the printing with high speed. Moreover, laser-assisted bioprinting is time-saving, costly within limited clinical applications (64).

Extrusion Bioprinting

Extrusion bioprinting delivers a continuous flow of bioink to the designed stage via a mechanical or pneumatic extrusion system. Most of the non-biological aims use this 3D printer system because of the wide spectrum of printing material among the other bioprinters. This bioprinter has the widest range of biomaterials such as hydrogels, copolymers, spheroids that have suitable viscosity for printing. While a pneumatic system uses compressed air to run bioink from the nozzle, mechanical system uses piston or screw that is controlled robotically. However one of the important disadvantages is that it needs more complex structure than pneumatic system.

On the other hand, there can be loss of cell viability for cell-loaded bioinks because of shear stress during the printing process. Scaffold can be affected by different factors such as viscosity and flow rate of bioink and nozzle shape and size (65).

Table 2.3 Advantages, Properties, and Limitations of 3D Printing Systems (65).

Bioprinting Techniques	Advantage	Limitation	Resolution	Speed
Inkjet	Ease of modification Low cost Simple operation Good resolution (μm~pm) and precision Fast printing speed Color printing Multi-material printing Can be printed onto non-flat surfaces Provision of concentration gradient	Bulky size Limited variety of bioink Low cell density Imposing thermal or acoustic stress to cells Clogging of nozzle Lack of effective structural integrity	<5 μm to 50 μm	1-10,000 droplets per second
Laser-assisted	Wide range of viscosity accommodated High cell viability High resolution Nozzle free and non-contact printing Precision up to nano-scale High cell density bioink	Limited control over printing directionality Metallic residual Possible damage of tissue from use of laser lights Limited number of photocrosslinking agents Slow printing speed Possibility of damage due to UV light Difficulty in handling heterogenous cells	>50 μm	200-1,600 mm/s
Extrusion	Wide selection of biomaterials (viscosity) High resolution	Low cell viability due to mechanical or shear stress Limited material selection (shear thinning ability required) Gelation and solidification Decreasing cell viability with increase in pressure	>5 μm	10-50 μm/s

2.2.4 3D Bioprintable Hydrogels for Bone Tissue Engineering

Hydrogels are biomaterials that are comprised of crosslinked hydrophilic polymers to compose three-dimensional (3D) networks. Since their structures mimic natural ECM, they are very useful for bone tissue engineering.

There are main hydrogels used in 3D bioprinting for bone tissue engineering as natural, such as hyaluronic acid, chitosan, cellulose, chitin, and alginate due to their suitable viscosity and gelatin for including RGD peptide or synthetic polymers such as poly(ethylene glycol) (PEG) derived polymers due to their mechanical strength (66).

There are many uses of these polymers in the literature. Noh and his colleagues proved the printability of hyaluronic acid-based bioink hydrogel consisting of hyaluronic acid, hydroxyethyl acrylate and gelatin-methacrylate via the mechanism of radical polymerization for bone tissue engineering. On the other hand, they provided the cell adhesion effect of gelatin due to RGD peptide (67).

In another study, Rogine et al. have shown the printability of chitosan by incorporating poly(lactic acid) (PLA) which is a synthetic polymer plays an important role in mechanical enhancement and hydroxyapatite to increase osteogenic properties (68). On the other hand, Kalkandelen and colleagues prepared gelatin/alginate/β-TCP composite bioink for 3D printing system and could be printed successfully. They demonstrated high cell viability and osteosarcoma cell adhesion by using β-TCP at different concentrations. They used gelatin for RGD cell adhesive peptide, Alginate due to its viscosity and β-TCP for osteosarcoma cell osteoconduction and osteoinduction (69).

On the other hand, there are different studies about the cell adhesion effects of KRSR peptide in literature. Balasundaram and his colleagues functionalized Titanium (Ti) surfaces for chemically immobilization of KRSR peptide onto materials (70). Moreover, they used the KSRR peptide as negative control. As a result, they showed that KRSR peptide has more cell adhesive effect than KSRR. In another study, Hoyos-Nogues and colleagues covalently bound KRSR peptide onto Titanium (Ti) surfaces by using the silanization method to improve the osteoblastic function of biomaterial (71). As a result, they showed that the KRSR peptide is osteoblast cell-adhesive.

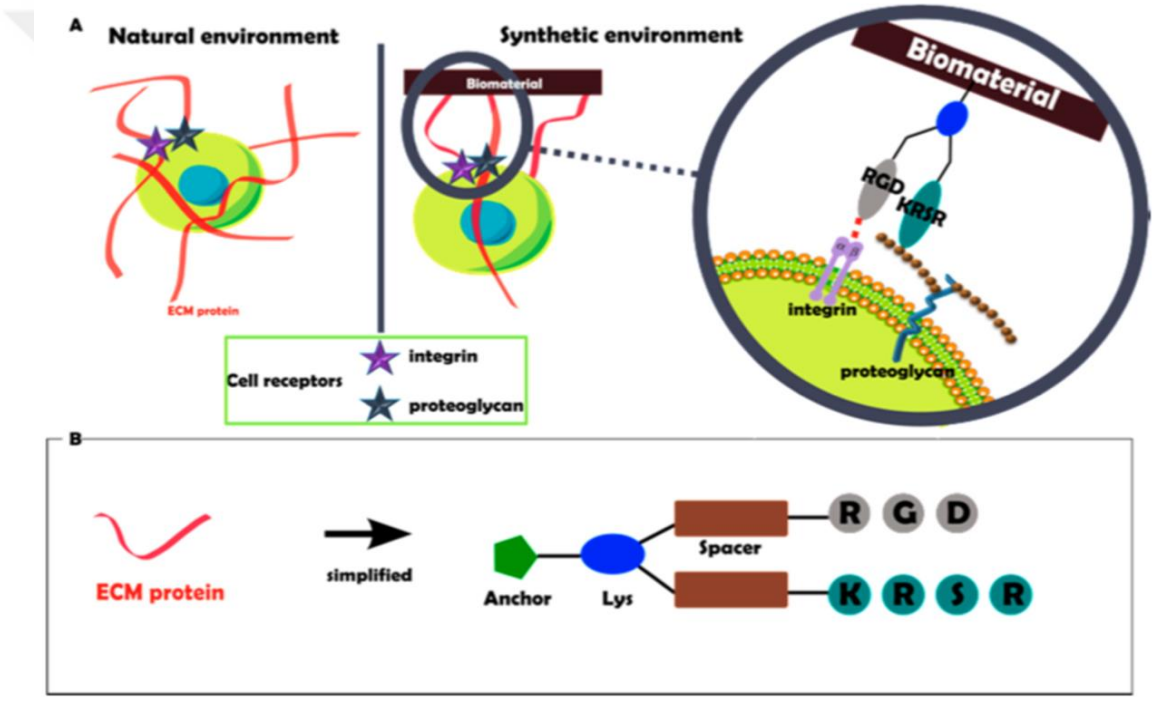

Figure 2.21 To stimulate and regulate cell behavior, native ECM proteins present in bone tissues include integrin and cell membrane heparan sulfate proteoglycan binding ligands (71).

3. MATERIALS AND METHODS

3.1. Materials

Alginic acid sodium salt from brown algae (medium viscosity- A2033), chitosan (HMW, 310-375 kDa), Gelatin (Type B, powder, BioReagent, suitable for cell culture), Hyaluronic acid sodium salt from Streptococcus equi (Bacterial Glycosaminoglycan polysaccharide), Methacrylic Anhydride (contains 2.000 ppm propanol A as inhibitor, 94%) were purchased from Sigma Aldrich for the synthesis methacrylate anhydride conjugated polymers. Poly(ethylene glycol) dimethacrylate (average Mn 750, contains 900-1100 ppm MEHQ as inhibitor), Irgacure 2959 (2-Hydroxy-4′-(2-hydroxyethyl)-2-methylpropiophenone) were purchased from Sigma Aldrich for preparation of crosslinked polymers. Carbonate Buffer was prepared from calcium carbonate and calcium bicarbonate (Sigma Aldrich) in distilled water to dissolve gelatin polymer. Acetic acid (absolute) was purchased from Merck to dissolve Chitosan polymer (used by diluting to 2 %). Aluminum oxide (90 active basics (0.063-0.200 mm) (activity stage I) for column chromatography) was purchased from Merck and used as a filter to remove inhibitor of Poly (ethylene glycol) dimethacrylate and SnakeSkin™ Dialysis Tubing (10 kDa) was purchased from Thermofisher.

For *in-vitro* experiments: dimethyl sulfoxide (DMSO, Sigma-Aldrich), fetal bovine serum (FBS, ATCC 30-2020™), penicillin-streptomycin (Pen/Strep, Gibco™), Dulbecco's modified Eagle's medium (DMEM, Gibco™) were purchased. Live/Dead Cell Viability Assay Kit (ab287858) was purchased from Abcam and Cell Counting Kit-8 (CCK-8) assay was purchased from GlpBio.

3.2. Preparation of 3D Printable Scaffold Systems

In this project, 3D printable biomaterial ink systems were composed for synthesizing methacrylated-natural polymer, crosslinking methacrylated-natural polymers and forming hydrogels from blended biomaterial bioinks, KRSR osteoblast adhesive peptide encapsulation into the scaffold and *in-vitro* evaluation of scaffolds.

3.2.1 Synthesizing of Methacrylated Natural Polymers

There are different types of natural polymers used in this project. These are methacrylated-gelatin/alginate/hyaluronic acid/chitosan. Each of them has its synthesizing method according to the literature. In this framework, all steps were mentioned above in the following steps. Finally, concentration of methacrylic anhydride bound to polymers was measured by using a UV-Vis spectrophotometer.

3.2.1.1. Synthesizing of Methacrylated Gelatin (Gel-MA) Polymers

Methacrylated Gelatin (Gel-MA) procedure was taken from the literature (72). First of all, dissolving solvent (carbonate buffer (CB)) of gelatin polymer was prepared based on literature. Briefly 0,318 gr sodium carbonate ($NaCO_3$) and 0,586 gr sodium bicarbonate ($NaHCO_3$) were dissolved in 100 mL (stock solution) ultrapure (Milli-Q) water as 0.1 percentage. In following step, 5 gr Gelatin powder was dissolved 50 mL CB (0,1 % w/v) by continuously stirring at 50 °C and 1500 rpm until homogeneously dissolved. 5 mL methacrylic anhydride was added to the solution dropwise at 80 °C, 1500 rpm for 3 hours under N_2 gas to form inert media. The reaction was adjusted to pH 8-9 by adding 5N NaOH to fix the acidity of the reaction.

Then, the solution was dialyzed against the deionized water for about 1 week through the 10 kDa dialysis membrane to filter small molecules smaller than 10 kDa such as unreacted methacrylic anhydride, methacrylic acid which are side products. After the dialysis process, the solution was stored at -80 °C for 3-4 h and finally lyophilized to obtain dry Gel-MA polymers.

3.2.1.2. Synthesizing of Methacrylated Chitosan (Chi-MA) Polymers

Methacrylated Chitosan (Chi-MA) procedure was taken from the literature (73). Briefly 0,5 gr Chitosan polymer (High molecular weigh) was dissolved in 50 mL 2 % (v/v) acetic solution by continuously stirring at 50 °C and 1500 rpm until homogeneously dissolved. 0,5 mL methacrylic anhydrite was added as dropwise to solution by continuously stirring at 80 °C and 1500 rpm for 4 hours under N_2 gas to form inert media. The reaction was fixed to pH 8-9 by adding 5N NaOH to adjust the pH of reaction. Then, the solution was dialyzed against the deionized water for about 1 week through the 10 kDa dialysis membrane to filter small molecules smaller than 10 kDa such as unreacted methacrylic anhydride, methacrylic acid which are side products. After the dialysis process, the solution was stored at -80 °C for 3-4 h and finally lyophilized to obtain dry Chi-MA polymers.

3.2.1.3. Synthesizing of Methacrylated Alginate (Al-MA) Polymers

Methacrylated Alginate (Al-MA) procedure was taken from the literature (74). Briefly 0,4 gr Alginic acid sodium salt was dissolved in 40 mL deionized water by continuously stirring at 50 °C and 1500 rpm until homogeneously dissolved. After being completely dissolved 3 mL methacrylic anhydrite was added as dropwise to solution by continuously stirring at 80 °C and 1500 rpm for 4 hours under N_2 gas to form inert media.

The reaction was fixed to pH 8-9 by adding 5N NaOH to adjust pH of reaction. Then, the solutions were dialyzed against the deionized water for about 1 week through the 10 kDa dialysis membrane to filter small molecules smaller than 10 kDa such as unreacted methacrylic anhydride, methacrylic acid which are side products. After the dialysis process, the solution was stored at -80 °C for 3-4 h and finally lyophilized to obtain dry Al-MA polymers.

3.2.1.4. Synthesizing of Methacrylated Hyaluronic Acid (Ha-MA) Polymers

Methacrylated Hyaluronic Acid (Ha-MA) procedure was taken from the literature (75). Briefly, 1 gr Hyaluronic acid sodium salt was dissolved in 100 mL of deionized water by continuously stirring at 50 °C and 1500 rpm until homogeneously dissolved. After being completely dissolved 7,5 mL methacrylic anhydride was added as dropwise to solution by continuously stirring at 80 °C and 1500 rpm for 4 hours under N_2 gas to form inert media. The reaction was fixed to pH 8-9 by adding 5N NaOH to adjust pH of reaction. Then, the solutions were dialyzed against the deionized water for about 1 week through the 10 kDa dialysis membrane to filter small molecules smaller than 10 kDa such as unreacted methacrylic anhydride, methacrylic acid which are side products. After the dialysis process, the solution was stored at -80 °C for 3-4 h and finally lyophilized to obtain dry Ha-MA polymers.

3.2.2 Crosslinking of Methacrylated-Natural Polymers

3.2.2.1 Preparing of Gelatin-Methacrylated (Gel-MA) Hydrogels

To prepare Gel-MA hydrogel, lyophilized Gel-MA prepolymer (10 % w/v) was dissolved in 1 mL deionized water and mixed with Irgacure 2959 (2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone) (2 % w/v) at 60 ºC until completely dissolved 1500 rpm. After being dissolved, it was put under ultraviolet light - UV (Analytic Jena US, 365 nm, 100 watts) for 20 minutes. Obtained and crosslinked Gel-MA hydrogel was washed with deionized water 3 times and stored at -80 ºC for 1-2 h. Finally lyophilized to obtain dry Gel-MA hydrogel and stored at 4 ºC.

3.2.2.2 Preparing of Chitosan-Methacrylated (Chi-MA) Hydrogels

To prepare Chi-MA hydrogel, lyophilized Chi-MA prepolymer (1 % w/v) was dissolved in 1 mL acetic acid (2 % v/v) and mixed with Irgacure 2959 (2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone) (0,15 % w/v) at 60 ºC until completely dissolved at 1500 rpm. After being dissolved, it was put under ultraviolet light (UV-365 nm) for 15 minutes. Obtained and crosslinked Chi-MA hydrogel was washed with deionized water 3 times and stored at -80 ºC for 1-2 h. Finally lyophilized to obtain dry Chi-MA hydrogel and stored at 4 ºC.

3.2.2.3 Preparing of Alginate-Methacrylated (Al-MA) Hydrogels

To prepare Al-MA hydrogel, lyophilized Al-MA prepolymer (3,5 % w/v) was dissolved in 1 mL deionized water and mixed with Irgacure 2959 (2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone) (2 % w/v) at 60 ºC until completely dissolved at 1500 rpm. After being dissolved, it was put under ultraviolet light (UV-365 nm) for 1 h. Obtained and crosslinked Al-MA hydrogel was washed with deionized water 3 times and stored at -80 ºC for 1-2 h. Finally lyophilized to obtain dry Al-MA hydrogel and stored at 4 ºC.

3.2.2.4 Preparing of Hyaluronic acid- Methacrylated (Ha-MA) Hydrogels

To prepare Ha-MA hydrogel, lyophilized Ha-MA prepolymer (2,5 % w/v) was dissolved in 1 mL deionized water and mixed with Irgacure 2959 (2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone) (2 % w/v) at 60 ºC until completely dissolved at 1500 rpm. After being dissolved, it was put under ultraviolet light (UV-365 nm) for 1 h. Obtained and crosslinked Ha-MA hydrogel was washed with deionized water 3 times and stored at -80 ºC for 1-2 h. Finally lyophilized to obtain dry Ha-MA hydrogel and stored at 4 ºC.

3.2.3 Preparation and 3D Bioprinting of Biomaterial Inks

In this step, different types of biomaterial formulations were prepared by using hyaluronic acid, alginate and chitosan. Prepared biomaterial inks were 3D printed by using (extrusion-based bioprinting) Axolotl Bioprinter Dual Print Head System (Axolotbio, Türkiye).

Syringes were filled with prepared biomaterial inks and printed with a speed of 0.25 mm/s, 22 G (22 Gauge = 0.644 mm) and 15.6 Pounds Per Square Inch (Psi) at room temperature. 3D printable inks were kept at -80 ºC for 1-2 h. Finally, lyophilized and dry hydrogels were stored at 4 ºC for the next characterizations.

3.2.3.1 Preparation and 3D Bioprinting of Biomaterial Inks with Hyaluronic Acid

Formulation 1: HA-HG-1

To prepare HA-HG-1, Hyaluronic acid sodium salt (4 % w/v) and Irgacure (0,9 % w/v) were dissolved in double distilled deionized water until completely dissolved and stirred at 60 ºC for 1-2 h until being dissolved at 1500 rpm. PEGdiMA (Poly(ethylene glycol)di Methacrylate) (10 % v/v) was added and continued for stirring until being homogeneous. After that, syringe of 3D bioprinter was filled with obtained biomaterial ink and crosslinked with UV for 1 min after printed and washed with double distilled water 3 times.

Formulation 2: HA-HG-2

To prepare HA-HG-2, Hyaluronic acid sodium salt (2 % w/v) and Irgacure (1 % w/v) were dissolved in double distilled deionized water until completely dissolved and stirred at 60 ºC for 1-2 h until being dissolved at 1500 rpm. PEGdiMA (Poly(ethylene glycol)di Methacrylate) (25 %v/v) was added and continued for stirring until being homogeneous. After that, the syringe of 3D bioprinter was filled with obtained biomaterial ink and crosslinked with UV for 1 min after printed and washed with double distilled water 3 times.

Formulation 3: HA-HG-3

To prepare HA-HG-3, Hyaluronic acid sodium salt (3 % w/v) and Irgacure (1 % w/v) were dissolved in double distilled deionized water until completely dissolved and stirred at 60 ºC for 1-2 h until being dissolved at 1500 rpm. PEGdiMA (Poly(ethylene glycol)di Methacrylate) (20 % v/v) was added and continued for stirring until being homogeneous. After that, syringe of 3D bioprinter was filled with obtained biomaterial ink and crosslinked with UV for 1 min after printed and washed with double distilled water 3 times.

Formulation 4: HA-HG-4

To prepare HA-HG-4, Gel-MA (5 % w/v) was dissolved in double distilled deionized water until completely dissolved. Then, Hyaluronic acid sodium salt (2,5 % w/v) and Irgacure (1 % w/v) were added and stirred at 60 ºC for 1-2 h until being dissolved at 1500 rpm. PEGdiMA (Poly(ethylene glycol)di Methacrylate) (25 % v/v) was added and continued for stirring until being homogeneous. After that, syringe of 3D bioprinter was filled with obtained biomaterial ink and crosslinked with UV for 1 min after printed and washed with double distilled water 3 times.

Formulation 5: HA-HG-5

To prepare HA-HG-5, Gel-MA (10 % w/v) was dissolved in double distilled deionized water until completely dissolved. Then, Hyaluronic acid sodium salt (2,5 % w/v) and Irgacure (2 % w/v) were added and stirred at 60 ºC for 1-2 h until being dissolved at 1500 rpm.

PEGdiMA (Poly(ethylene glycol)di Methacrylate) (25 % v/v) was added and continued for stirring until being homogeneous. After that, syringe of 3D bioprinter was filled with obtained biomaterial ink and crosslinked with UV for 1 min after printed and washed with double distilled water 3 times.

Formulation 6: HA-HG-6

To prepare HA-HG-6, Al-MA (2 % w/v) was dissolved in double distilled deionized water until completely dissolved. Then, Hyaluronic acid sodium salt (2 % w/v) and Irgacure (2 % w/v) were added and stirred at 60 ºC for 1-2 h until being dissolved at 1500 rpm. PEGdiMA (Poly(ethylene glycol)di Methacrylate) (30 % v/v) was added and continued for stirring until being homogeneous. After that, syringe of 3D bioprinter was filled with obtained biomaterial ink and crosslinked with UV for 1 min after printed and washed with double distilled water 3 times.

3.2.3.2 Preparation and 3D Bioprinting of Biomaterial Ink with Chitosan

Formulation 1: CH-HG-1

To prepare CH-HG-1, Chitosan (High Molecular Weight) (2,5 % w/v) and Irgacure (2 % w/v) were dissolved in acetic acid solution (2 % v/v) until completely dissolved at 1500 rpm. PEGdiMA (Poly(ethylene glycol)di Methacrylate) (25 % v/v) was added and continued for stirring until being homogenous. After that, syringe of 3D bioprinter was filled with obtained biomaterial ink and crosslinked with UV for 1 min after printed and washed with double distilled water 3 times.

Formulation 2: CH-HG-2

To prepare CH-HG-2, Gel-MA (3 % w/v) was dissolved in double distilled deionized water until completely dissolved. Then, chitosan (High Molecular Weight) (1,5 % w/v) and Irgacure (2 % w/v) were added and stirred at 60 ºC for 1-2 h until being dissolved at 1500 rpm. PEGdiMA (Poly(ethylene glycol)di Methacrylate) (25 % v/v) was added and continued for stirring until being homogeneous. After that, syringe of 3D bioprinter was filled with obtained biomaterial ink and crosslinked with UV for 1 min after printed and washed with double distilled water 3 times.

Formulation 3: CH-HG-3

To prepare CH-HG-3, Gelatin (Type B, powder, BioReagent) (2 % w/v) was dissolved in acetic acid (2 % v/v) solution until completely dissolved. Then, chitosan (High Molecular Weight) (2 % w/v) and Irgacure (2 % w/v) were added and stirred at 60 ºC for 1-2 h until being dissolved at 1500 rpm. PEGdiMA (Poly(ethylene glycol)di Methacrylate) (25 % v/v) was added and continued for stirring until being homogeneous. After that, syringe of 3D bioprinter was filled with obtained biomaterial ink and crosslinked with UV for 1 min after printed and washed with double distilled water 3 times.

Formulation 4: CH-HG-4

To prepare CH-HG-4, Gelatin (Type B, powder, BioReagent) (% 10 w/v) was dissolved in acetic acid (2 % v/v) solution until completely dissolved. Then, chitosan (High Molecular Weight) (1,5 % w/v) and Irgacure (2 % w/v) were added and stirred at 60 ºC for 1-2 h until being dissolved at 1500 rpm.

PEGdiMA (Poly(ethylene glycol)di Methacrylate) (25 % v/v) was added and continued for stirring until being homogenous. After that, syringe of 3D bioprinter was filled with obtained biomaterial ink and crosslinked with UV for 1 min after printed and washed with double distilled water 3 times.

Formulation 5: CH-HG-5

To prepare CH-HG-5, Gel-MA (10 % w/v) was dissolved in double distilled deionized water until completely dissolved. Then, chitosan (High Molecular Weight) (2 % w/v) and Irgacure (2 % w/v) were added and stirred at 60 ºC for 1-2 h until being dissolved at 1500 rpm. PEGdiMA (Poly(ethylene glycol)di Methacrylate) (25 % v/v) was added and continued for stirring until being homogeneous. After that, syringe of 3D bioprinter was filled with obtained biomaterial ink and crosslinked with UV for 1 min after printed and washed with double distilled water 3 times.

Formulation 6: CH-HG-6

To prepare CH-HG-6, Chi-MA (2,5 % w/v) and Gelatin (10 % w/v) were dissolved in carbonate buffer until completely dissolved and stirred at 60 ºC for 1-2 h until being dissolved at 1500 rpm. PEGdiMA (Poly(ethylene glycol)di Methacrylate) (25 % v/v) and Irgacure (2 % w/v) were added and continued for stirring until being homogeneous. After that, syringe of 3D bioprinter was filled with obtained biomaterial ink and crosslinked with UV for 1 min after printed and washed with double distilled water 3 times.

3.2.3.3 Preparation and 3D Bioprinting of Biomaterial Ink with Alginate

Formulation 1: AL-HG-1

To prepare AL-HG-1, Alginic acid sodium salt from brown algae (medium viscosity) (4 % w/v) was dissolved in double distilled deionized water until completely dissolved and stirred at 60 ºC for 1-2 h until being dissolved at 1500 rpm. PEGdiMA (Poly(ethylene glycol)di Methacrylate) (20 % v/v) and Irgacure (1,5 % w/v) were added and continued for stirring until being homogeneous. After that, syringe of 3D bioprinter was filled with obtained biomaterial ink and crosslinked with UV for 1 min after printed and washed with double distilled water 3 times.

Formulation 2: AL-HG-2

To prepare AL-HG-2, Alginic acid sodium salt from brown algae (medium viscosity) (4 % w/v) was dissolved in double distilled deionized water until completely dissolved and stirred at 60 ºC for 1-2 h until being dissolved at 1500 rpm. PEGdiMA (Poly(ethylene glycol)di Methacrylate) (10 % v/v) and Irgacure (1,8 % w/v) were added and continued for stirring until being homogeneous. After that, syringe of 3D bioprinter was filled with obtained biomaterial ink and crosslinked with UV for 1 min after printed and washed with double distilled water 3 times.

3.2.4 Characterization of Hydrogels

Swelling Test: Hydrogels dried by the lyophilization method were incubated in distilled water at room temperature, removed and weighed at certain time intervals (0, 5, 10, 15…40 min) and compared in percentage with the weight of their first dry state. Thus, the time-dependent water uptake capacity of hydrogels was calculated as a percentage (76). Swelling is calculated by given formula:

$$\% \text{ Swelling Ratio: } \frac{Ws-Wd}{Wd} \times 100 \qquad \text{(Eq. 3.1)}$$

Ws: Swollen Gel

Wd: Dry Gel

Rheological evaluation: The rheometer (Kinexus Malvern) was used to analyze the viscoelastic behavior of synthesized hydrogels by subjecting a sample to various types of stress such as shear stress, oscillation and deformation with J2 SR 4703 SS plate-plate geometry. The deformation degrees of the hydrogels to be obtained in this thesis were analyzed both at room temperature by applying shear stress in oscillation mode (77). G' and G" values were measured for biomaterial inks swollen in distilled water and at +4 °C overnight, and a comparison was made between biomaterial inks in terms of deformation and durability. G’ and G’’ values were evaluated for all hydrogels between 10^1-10^5 and shear strain γ was evaluated between 10^{-2}-10^3 at 1 Hz frequency, 25 ºC and 1 mm gap, at 25 ºC.

Functional Group Analysis: Characteristic functional groups belonging to the chemical structures of the molecules within the lyophilized hydrogel structures were determined using Fourier Transform Infrared Spectroscopy (FT-IR) (Thermo Fisher Scientific Inc. Nicolet 380) (78).

Morphological analysis: The porosity and pore sizes of the hydrogel scaffolds, which are expected to have a porous structure, were monitored with Scanning Electron Microscope (SEM) (Thermo-Fisher Scientific Quattro S ESEM). Dry hydrogel samples with gold (Au) coating were observed with a high vacuum detector (79).

Gel Conversion: This characterization method was performed to determine how much viscous gel is converted to crosslinked gel after drying.

$$\% \text{ Gel Conversion: } \frac{\text{Obtained amount after drying}}{\text{Starting amount of all components}} \times 100$$

(Eq. 3.2)

Measurement of Methacrylation Ratio of Hydrogels: This characterization method was performed to determine how much methacrylate groups are attached to the side chains of the methacrylated natural polymers. The degree of methacrylation was measured

quantitatively using an UltraViole-Visible (UV-Vis) spectrophotometer (Shimadzu UV-VIS 2600). A calibration curve was prepared for the methacrylate group using Poly(ethylene glycol) monomethyl ether methacrylate (PEGMEMA) monomer, which contains a single methacrylate in its structure and whose chain part consists of ethylene glycol molecules that do not absorb at 234 nm, where the methacrylate group gives maximum absorption (80). By using this calibration curve to be prepared at 234 nm, the methacrylation degrees of the polymers to which methacrylate was added to their structure were calculated as their weight ratio to the polymer.

3.2.5 Solid Phase Peptide Synthesis of KRSR

KRSR peptide was synthesized with The Liberty Blue^{TM} Automated Microwave Peptide Synthesizer (CEM). The synthesis scale was determined as 0,1 mmole.

KRSR peptide sequence consists of Lysine (Lys, K), Arginine (Arg, R) and Serine (Ser, S) used in this peptide synthesis in L form. Briefly, 0,29 grams of Lysine was dissolved in 3 mL of N, N-dimethylformamide (DMF). 1,56 grams of Arginine was dissolved in 12 mL of DMF and 0,24 grams of Serine was dissolved in 3 mL of DMF. N,N'-Diisopropylcarbodiimide (DIC) was used as the activator and Oxyma was used as the activator base. 0,6 mL of DIC was prepared with 7,4 mL DMF and 0,568 grams of Oxyma was prepared in 4 mL of DMF. The deprotection cocktail was prepared with 5 mL Piperadine in 20 mL DMF.

At the end of the peptide synthesize, resin has to be cleaved from the peptide. The device that was used to cleave resin from peptide is called the Razor™ Peptide Cleavage System (CEM). The cleavage cocktail that was used for this device's action consists of 4,75 mL of trifluoroacetic acid (TFA), 125 µL of triisopropylsilane (TIS) and 125 µL of double distilled water (ddH_2O). The peptide cleavage was carried out at 38 °C for 30 minutes. The cleaved peptide was precipitated in cold diethyl ether overnight at -20 °C and then centrifuged at 5000 rpm for 3 minutes. After centrifugation, diethyl ether and cleavage cocktail were discarded and repeated 3 times with cold diethyl ether and then the peptide pellet was dried by vacuum.

3.2.6 Characterization of Synthesized Peptide

3.2.6.1 High Pressure Liquid Chromatography-HPLC

The synthesized peptide was analyzed by using High Pressure Liquid Chromatography-HPLC (260 Infinity Quaternary LC systems, Agilent Technologies). First of all, the peptide (1 mg/mL) was dissolved in double distilled water (ddH_2O). Solutions which are ddH_2O/ Trifluoroacetic acid (TFA-0,05 %) and B solution acetonitrile (Acn)/Trifluoroacetic acid (TFA- 0,025 %) were used for the mobile phases as A and B at 2 mL/min flow rate and 214 nm and 280 nm were used for detection peaks at 25 ºC.

Table 3.1 HPLC method used for analysis of KRSR peptide.

Time (min)	Solution B (%)*
0-1	0
1-31	0-100
31-33	100

*Solvent B: Acetonitrile

3.2.7 *In-vitro* Cytotoxicity Assay

This test was used to evaluate toxicity for 4 chosen biomaterial ink hydrogels by using Cell Counting Kit-8 (CCK-8) viability assay against mouse fibroblast cell line (L929, ATCC CCL-1). Dulbecco's Modified Eagle Medium with phenol red (DMEM) was used as cell medium and completed with 10% fetal bovine serum (FBS) and 1% Pen/Strep antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin). Cells were incubated with completed DMEM at 37 °C and 5 % CO_2 until 80-90 % confluency was reached. After that, medium was removed and washed with 1X phosphate buffer saline (PBS), trypsinized 0,25% trypsin-EDTA solution and then incubated at 37°C for 5 min. After incubation, trypsin-EDTA was deactivated with DMEM and unattached cells were collected to falcon and centrifuged at 400 G for 5 min. The supernatant was removed and the pellet was resuspended with fresh medium. 10 µL cell solution was stained with trypan blue (1:1 ratio) and counted with a thoma hemocytometer. The formulation is to count the cells as follows:

$$\text{Cells (cell/mL)} : \text{Counted Cells} \times \text{Dilution Factor} \times 10^4 \quad \ldots\ldots\ldots\ldots \text{Equation (3.3)}$$

After the cell counting process, cells ($10x10^4$/well) were seeded to a 96-well plate with 100 μL complete DMEM and incubated at 37 °C for 24 hours for cells to adhere completely. Lyophilized biomaterial inks were weighed 10 mg of each and sterilized by UV light in a biosafety cabinet for almost 90 minutes. After sterilization, incubated (immersed) in 1 mL sterile cell medium for 2 sets at 3h and 6h at 37 °C by shaking. After incubation, immersed cell mediums were diluted with Dulbecco's phosphate buffer saline (DPBS) in as 1:5 ratio. Cell treatment was performed as indirectly method. Cells were treated with these diluted immersed cell medium and incubated at 37 °C for 24 hours. After 24h, the number of viable cells was determined by CCK-8 cell viability assay by adding 10 μL CCK-8 reagent in 100 μL in immersed medium onto wells and at the end of 2 hours incubation absorbance values at 450 nm were measured via a microplate reader. All experiments were performed in triplicates (n=3). Cytotoxicity values were calculated in excel.

3.2.8 Cell Adhesion Test

This test was evaluated to determine cell behavior for 4 chosen biomaterial ink hydrogels HA-HG-5, CH-HG-5, CH-HG-1 and CH-HG-3 by using Live/Dead Cell Viability Assay Kit (ab287858) against human fetal osteoblast cell line (hFOB 1.19, ATCC CRL-3602).

First of all, biomaterial inks were prepared in 0,2 mm thick circular disk and crosslinked under UV for 1 min. Then, these disks were placed into 24 well-plate and the excesses were trimmed. Hydrogels were washed with double distilled deionized water 3 times. Finally, hydrogel disks were sterilized by UV light in biosafety cabinet for almost 90 minutes.

For the cell adhesion, Dulbecco's Modified Eagle Medium (DMEM) was used as cell medium and completed with 10% fetal bovine serum (FBS) and 1% Pen/Strep antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin). Cells were incubated with completed DMEM at 37 °C and 5 % CO_2 until 80-90 % confluency was reached. After that, the medium was removed, washed with 1X phosphate buffer saline (PBS) and trypsinized 0,25% trypsin-EDTA solution, then incubated at 37°C for 5 min. After incubation, trypsin-EDTA was deactivated with DMEM and unattached cells were collected to a falcon and centrifuged at 1200 rpm for 5 min. The supernatant was removed and pellet was resuspended with fresh medium. 10 μL cell solution was taken and stained with trypan blue (1:1 ratio) and counted with thoma hemocytometer.

After cell counting process, cells ($10x10^4$/well) were seeded to the 24-well plate with hydrogel disks with 500 μL complete DMEM and incubated at 37 °C and 24 hours for cells to adhere completely. After incubation, the medium was removed and washed with DPBS and then the Live/Dead cell stain was suspended with DPBS at 5X concentration and added to the cells. Waited at dark and room temperature for 10 minutes. Stained as live (green) and dead (red) hFOB 1.19 cells adhered hydrogel disks were visualized with fluorescence microscope (EVOS™ M5000 Cell Imaging System, ThermoFisher).

3.2.9 Preparation of Peptide Encapsulated Biomaterial Inks

KRSR peptide was encapsulated for 2 chosen biomaterial inks which are HA-HG-5 and CH-HG-5.

Formulation HA-HG-5:

To prepare peptide encapsulated HA-HG-5, KRSR peptide (0,15 % w/v) dissolved in double distilled deionized water. After that, Gel-MA (10 % w/v) was added to aqueous peptide solution and stirred until completely dissolved. Then, hyaluronic acid sodium salt (2,5 % w/v) and Irgacure (2 % w/v) were added and stirred for 1-2 h until being dissolved at room temperature at 1500 rpm. PEGdiMA (Poly(ethylene glycol)di Methacrylate) (25 % v/v) was added and continued for stirring until being homogeneous.

Formulation CH-HG-5

To prepare peptide encapsulated CH-HG-5, KRSR peptide (0,15 % w/v) dissolved in 2 % acetic acid solution. After that, Gel-MA (10 % w/v) was added to the aqueous peptide solution and stirred until completely dissolved. Then, chitosan (High Molecular Weight) (2 % w/v) and Irgacure (2 % w/v) were added and stirred for 1-2 h until being dissolved at room temperature at 1500 rpm. PEGdiMA (Poly(ethylene glycol)di Methacrylate) (25 % v/v) was added and continued for stirring until being homogeneous.

3.2.10 Cell Adhesion Test for Peptide-Encapsulated Biomaterial Inks

This test was evaluated to determine cell behavior on the hydrogels by using Live/Dead Cell Viability Assay Kit (ab287858) against the human fetal osteoblast cell line (hFOB 1.19, ATCC CRL-3602).

First of all, peptide encapsulated biomaterial inks were prepared in 0,2 mm thick circular disk and crosslinked under UV for 1 min. Then, these disks were placed into 24 well-plate and the excesses were trimmed. Peptide encapsulated and crosslinked hydrogels were washed with double distilled deionized water 3 times. Finally, hydrogel disks were sterilized by UV light in biosafety cabinet for almost 90 minutes.

For the cell adhesion, Dulbecco's Modified Eagle Medium (DMEM) was used as cell medium and completed with 10 % fetal bovine serum (FBS) and 1 % Pen/Strep antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin). Cells were incubated with completed DMEM at 37 °C and 5 % CO_2 until 80-90 % confluency was reached. After that, medium was removed, washed with 1X phosphate buffer saline (PBS) and trypsinized 0,25 % trypsin-EDTA solution and then incubated at 37°C for 5 min. After incubation, trypsin-EDTA was deactivated with DMEM and unattached cells were collected to a falcon and centrifuged at 1200 rpm for 5 min. The supernatant was removed and the pellet was resuspended with fresh medium. 10 µL cell solution was taken and stained with trypan blue (1:1 ratio) and counted with a thoma hemocytometer.

After cell counting process, cells (100,000/well) were seeded to 24-well plate with hydrogel disks with 500 µL complete DMEM and incubated at 37 °C and 24 hours for cells to adhere completely. After incubation, medium was removed and washed with DPBS and then the Live/Dead cell stain was suspended with 1X PBS at 5x concentration and added to the cells. After that, waited at dark and room temperature for 10 minutes. Stained as live (green) and dead (red) hFOB 1.19 cells

adhered hydrogel disks were visualized with fluorescence microscope (EVOS^{TM} M5000 Cell Imaging System, ThermoFisher).

3.2.11 Alizarin Red S Staining

Mineralization of degree of hFOB 1.19 was evaluated for HA-HG-5 biomaterial ink formulation by using Alizarin Red S (Merck, Darmstadt, Germany) staining. The biomaterial ink was prepared with and without the KRSR peptide in 0,2 mm circular disk to demonstrate the effect of the KRSR peptide on mineralization. Before the staining process, hFOB 1.19 cell and hFOB 1.19 loaded hydrogel disks were incubated with 500 µL DMEM and 250 µL DPBS mixing for 14 days. At the end of the 14 days, hFOB 1.19 cells were fixed with a 4 % Paraformaldehyde (PFA) solution. After that, hFOB 1.19 cells were stained with 2 % Alizarin Red S for 1 hour, hFOB 1.19 loaded hydrogel disks were stained with 2 % Alizarin Red S for 30 minutes and washed with ddH_2O. Finally, stained cells were visualized with a fluorescence microscope (EVOS^{TM} M5000 Cell Imaging System, ThermoFisher).

4. RESULTS

4.1 Synthesizing Methacrylated-Natural Polymers

Natural polymers were reacted with methacrylic anhydrite. During the reaction, the pH of the solution was controlled with the pH strips. Finally waited for 24 h under N_2 gas to form inert media.

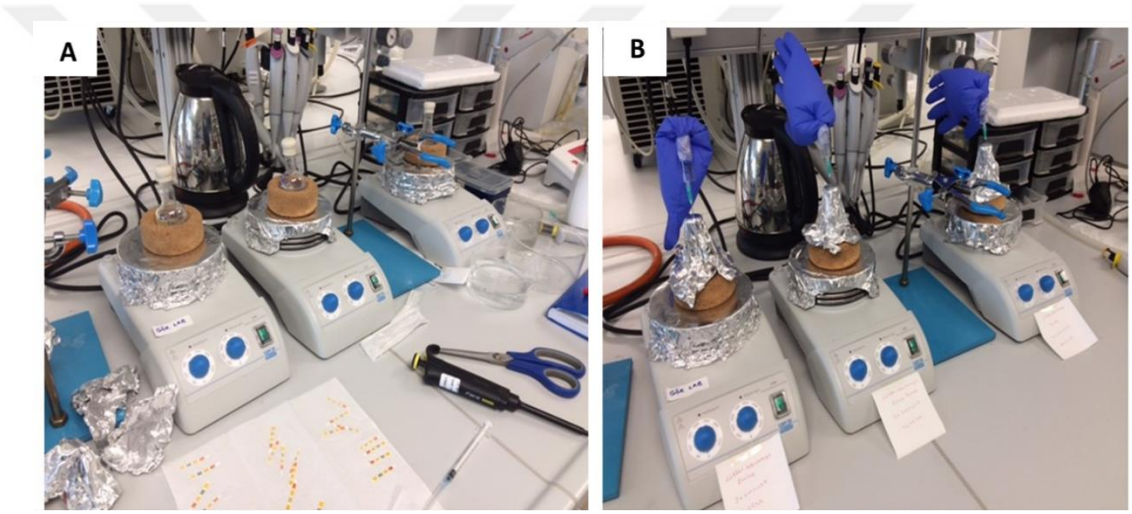

Figure 4.1 Synthesizing of Methacrylated-Natural Polymers and pH adjusting.

After the reaction, methacylated natural polymers were dialyzed for purification against the double distilled water for 1 week.

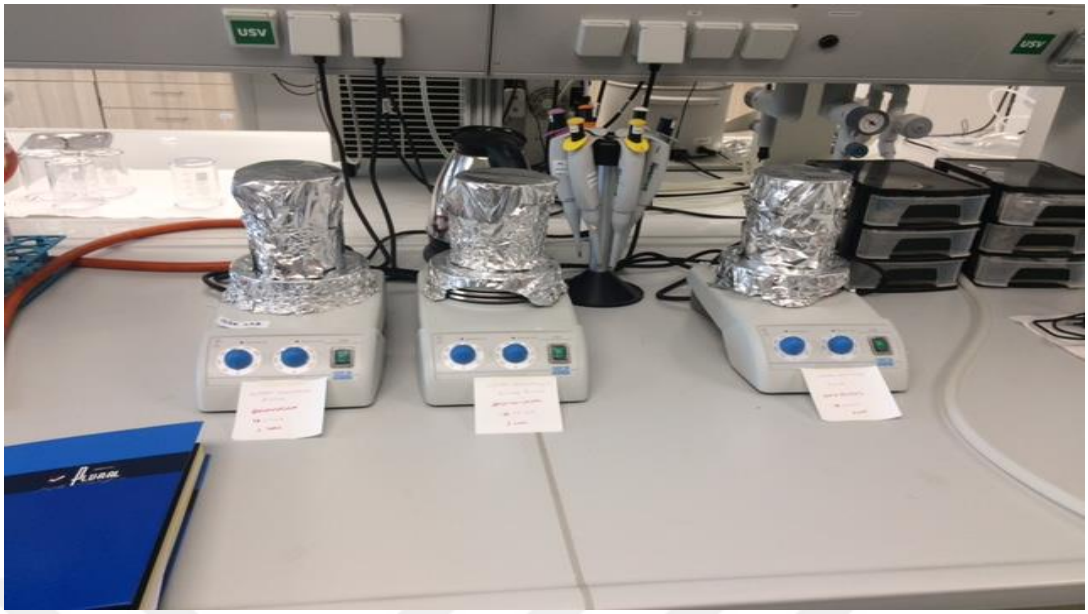

Figure 4.2 Dialyzing of Methacrylated-Natural Polymers.

Purificated methacrylated natural polymers were lyophilized and stored at +4 C.

Figure 4.3 After Lyophilization of Methacrylated-Natural Polymers.

4.2 Gellation Behaviours of Methacrylated-Natural Polymers

4.2.1. Preparing Gelatin-Methacrylated (Gel-MA) Hydrogels

Lyophilized Gel-MA was dissolved in ddH_2O and mixed with Irgacure (photoinitiator) and crosslinked in the existence of UV (365 nm).

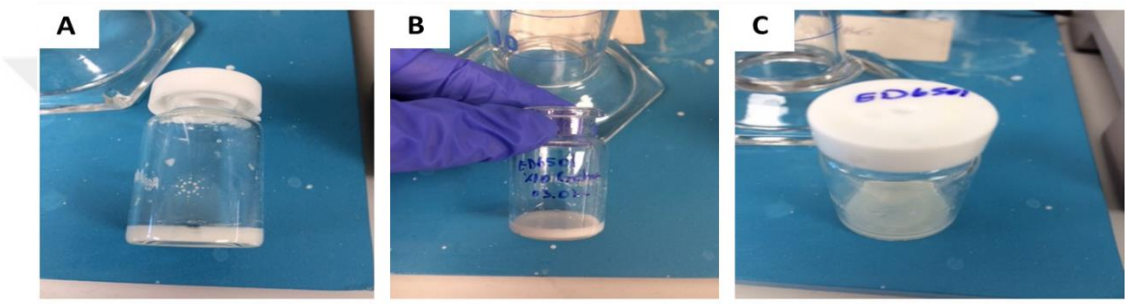

Figure 4.4 UV crosslinked Gel-MA hydrogels.

4.2.2. Preparing Chitosan-Methacrylated (Chi-MA) Hydrogels

Lyophilized Chi-MA was dissolved in ddH_2O and mixed with Irgacure (photoinitiator) and crosslinked in the existence of UV (365 nm).

Figure 4.5 UV crosslinked Chi-MA hydrogels.

4.2.3 Preparing Alginate-Methacrylated (Al-MA) Hydrogels

Lyophilized Al-MA was dissolved in ddH_2O and mixed with Irgacure (photoinitiator) and crosslinked in the existence of UV (365 nm).

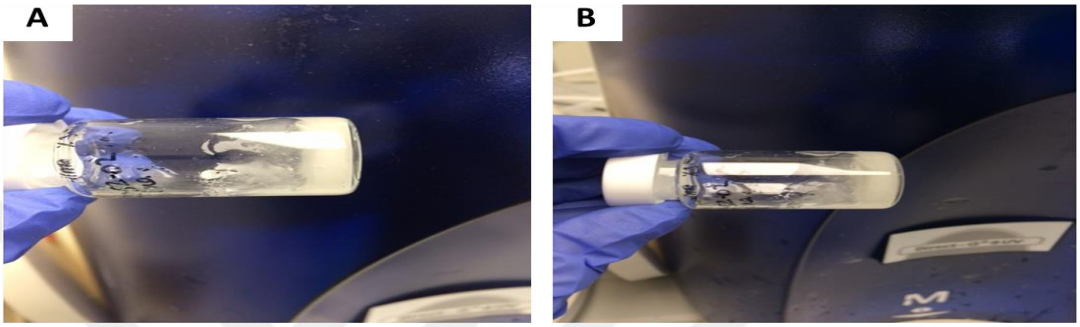

Figure 4.6 UV crosslinked Chi-MA hydrogels.

4.2.4 Preparing Hyaluronic Acid-Methacrylated (Ha-MA) Hydrogels

Lyophilized Ha-MA was dissolved in ddH_2O and mixed with Irgacure (photoinitiator) and crosslinked in the existence of UV (365 nm).

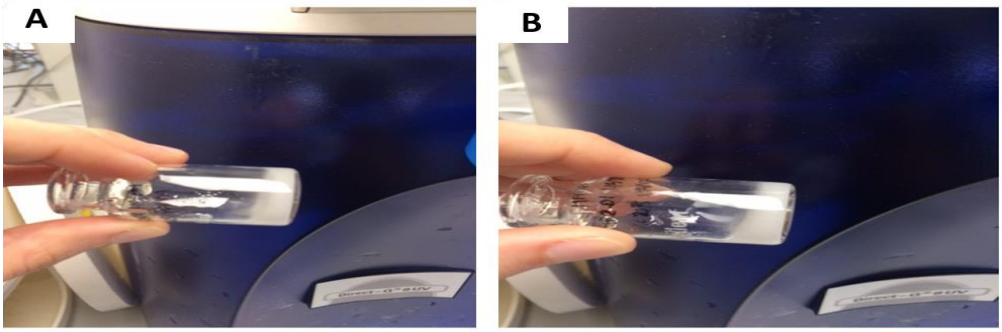

Figure 4.7 UV crosslinked Ha-MA hydrogels.

4.3 Preparation and 3D Bioprinting of Biomaterial Inks

Biomaterial Ink formulations were optimized using a 3D bioprinter.

4.3.1 3D Bioprinting of Biomaterial Inks with Hyaluronic Acid

Formulation 1: HA-HG-1

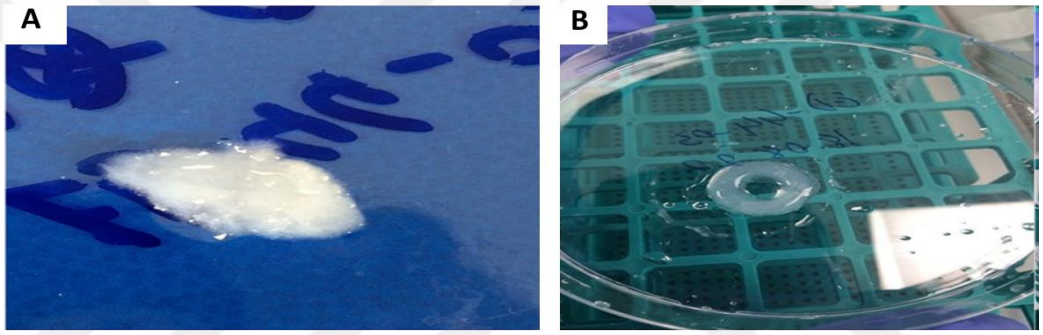

Figure 4.8 3D printing of HA-HG-1: Hyaluronic acid (4 % w/v) + PEGdiMA (10 % v/v) + Irgacure (0,9 % w/v).

Formulation 2: HA-HG-2

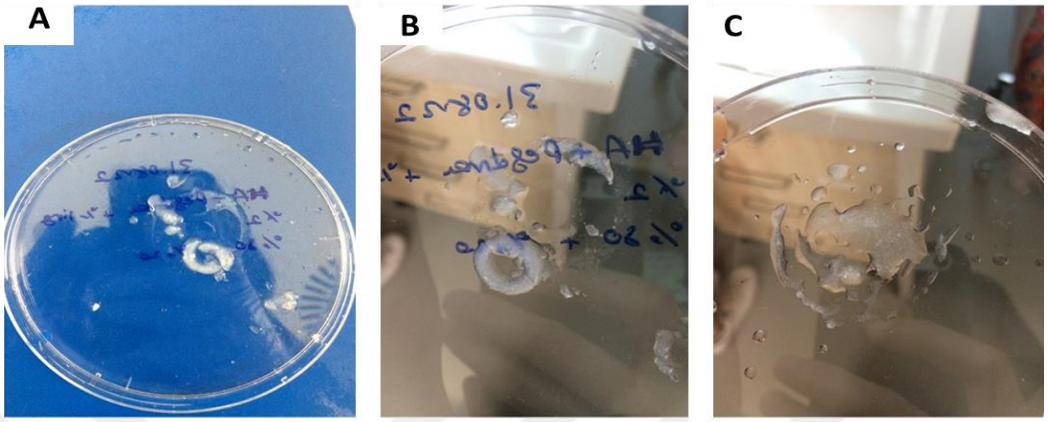

Figure 4.9 3D printing of HA-HG-2: Hyaluronic acid (2 % w/v) + PEGdiMA (25% v/v) + Irgacure (1 % w/v).

Formulation 3: HA-HG-3

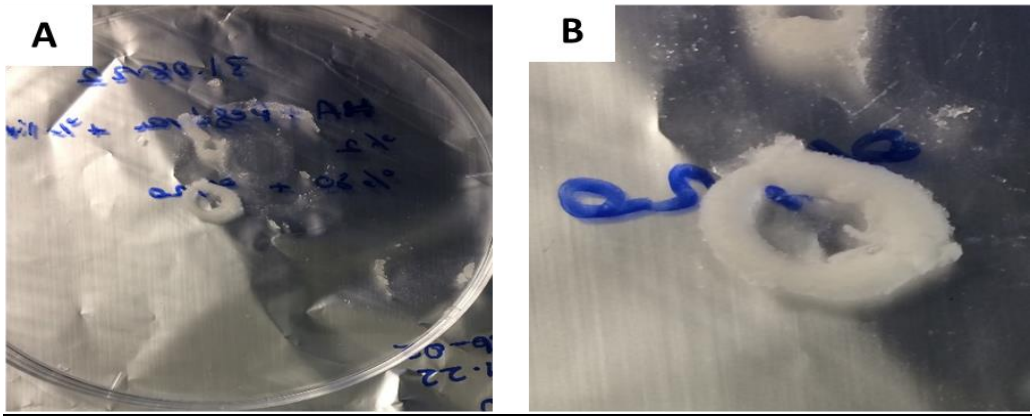

Figure 4.10 3D printing of HA-HG-3: Hyaluronic acid (3% w/v) + PEGdiMA (20% v/v) + Irgacure (1% w/v).

Formulation 4: HA-HG-4

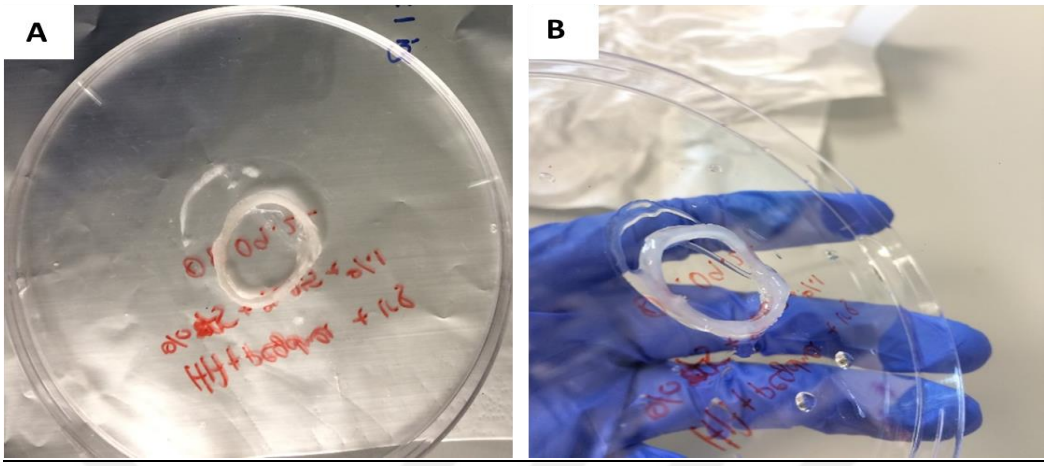

Figure 4.11 3D printing of HA-HG-4: Hyaluronic acid (2,5 % w/v) + Gel-MA (5 % w/v) + PEGdiMA (25 % v/v) + Irgacure (1 % w/v).

Formulation 5: HA-HG-5

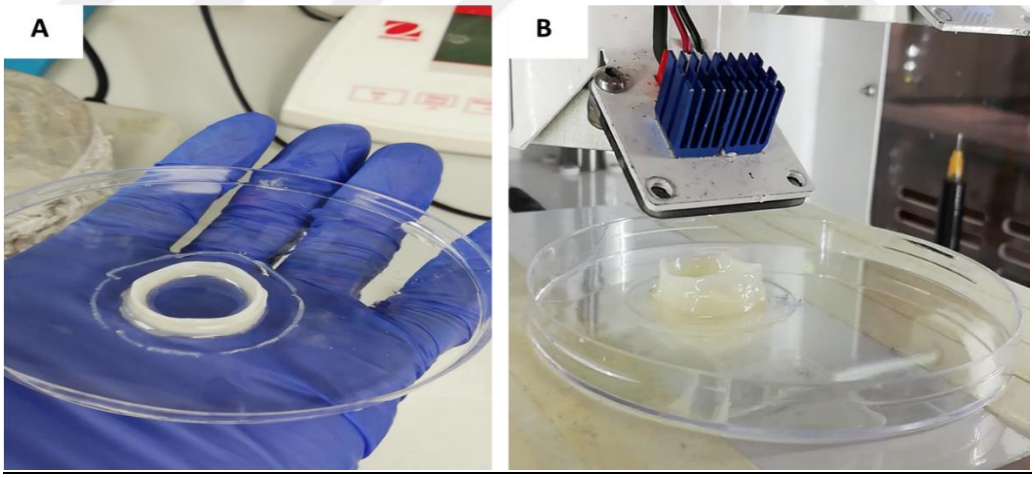

Figure 4.12 3D printing of HA-HG-5: Hyaluronic acid (2,5 % w/v) + Gel-MA (10 % w/v) + PEGdiMA (25 % v/v) + Irgacure (2 % w/v).

Formulation 6: HA-HG-6

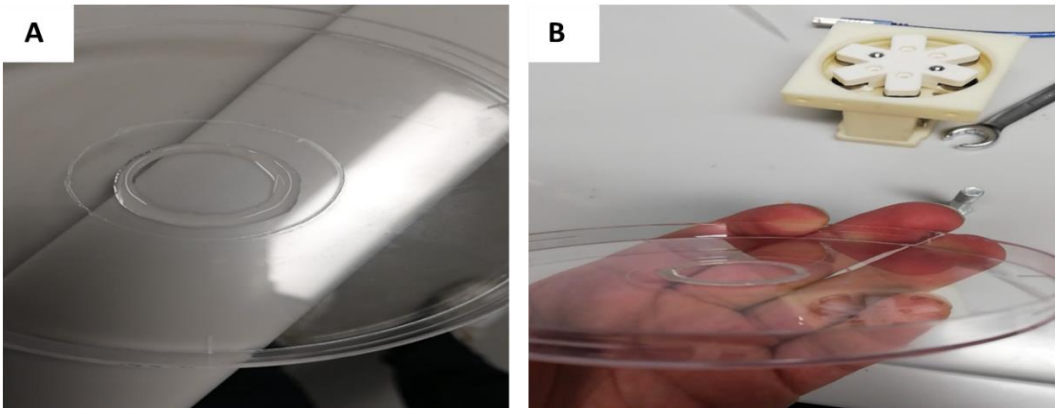

Figure 4.13 3D printing of HA-HG-6: Hyaluronic acid (2 % w/v) + Al-MA (2% w/v) + PEGdiMA (30 % v/v) + Irgacure (2 % w/v).

4.3.2 3D Bioprinting of Biomaterial Inks with Chitosan

Formulation 1: CH-HG-1

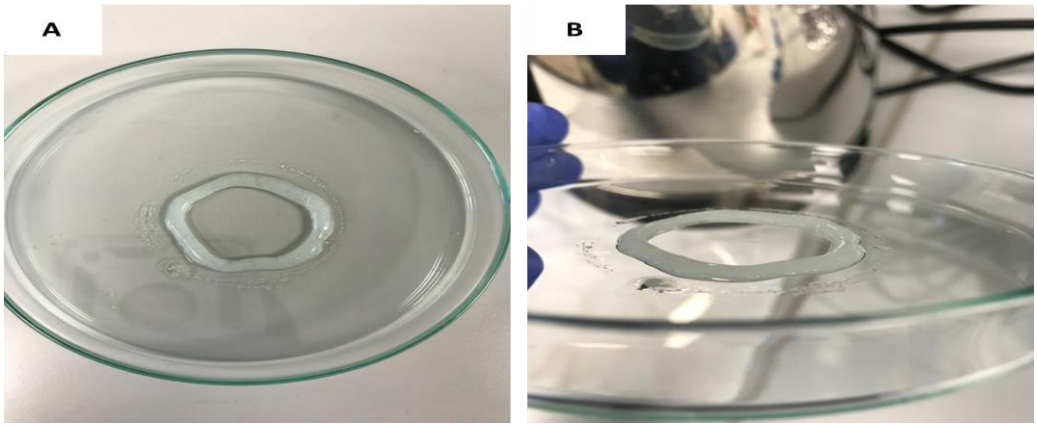

Figure 4.14 3D printing of CH-HG-1: Chitosan (2,5% w/v) + PEGdiMA (25% v/v) + Irgacure (2% w/v).

Formulation 2: CH-HG-2

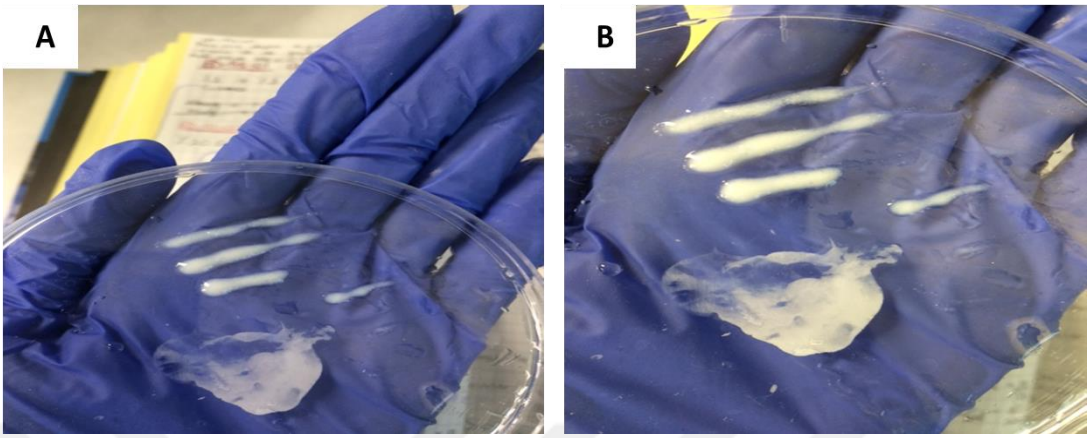

Figure 4.15 3D printing of CH-HG-2: Chitosan (1,5% w/v) + Gel-MA (3% w/v) + PEGdiMA (25% v/v) + Irgacure (2% w/v).

Formulation 3: CH-HG-3

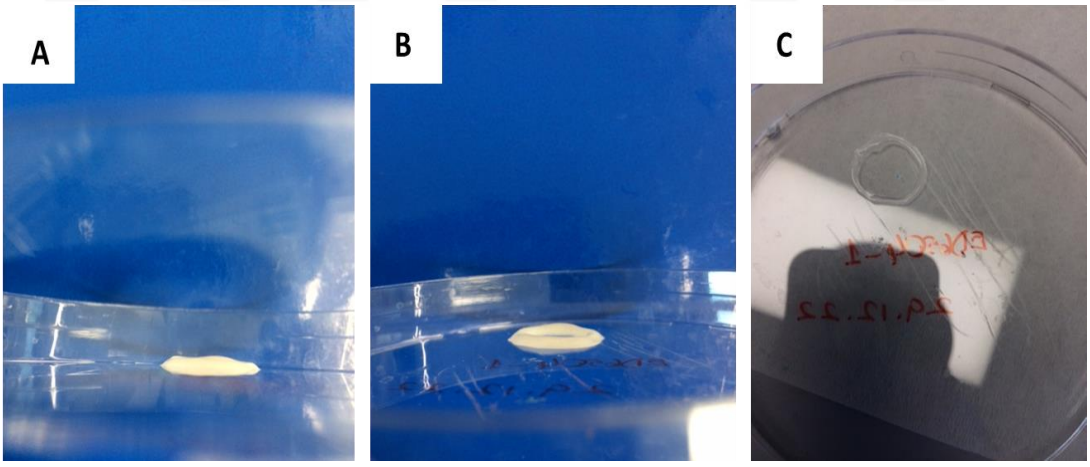

Figure 4.16 3D printing of CH-HG-3: Chitosan (2% w/v) + Gelatin (2% w/v) + PEGdiMA (25% v/v) + Irgacure (2% w/v).

Formulation 4: CH-HG-4

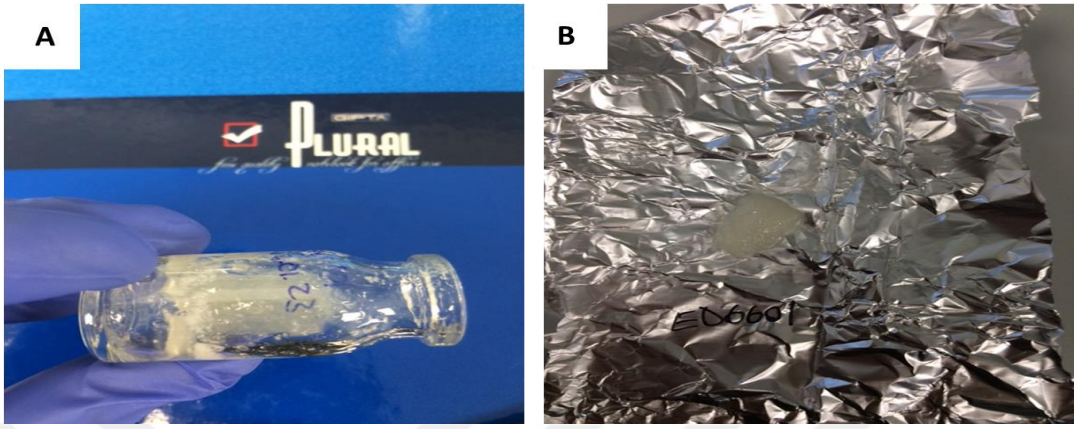

Figure 4.17 3D printing of CH-HG-4: Chitosan (1,5% w/v) + Gelatin (10% w/v) + PEGdiMA (25% v/v) + Irgacure (2% w/v).

Formulation 5: CH-HG-5

Figure 4.18 3D printing of CH-HG-5: Chitosan (2% w/v) + Gel-MA (10% w/v) + PEGdiMA (25% v/v) + Irgacure (2% w/v).

Formulation 6: CH-HG-6

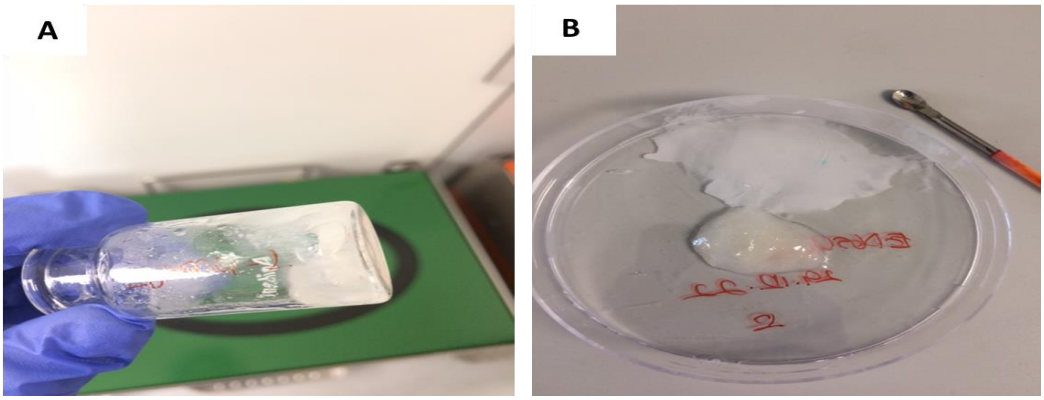

Figure 4.19 3D printing of CH-HG-6: Chi-MA (2,5% w/v) + Gelatin (10% w/v) + PEGdiMA (25% v/v) + Irgacure (2% w/v).

4.3.3 3D Bioprinting of Biomaterial Ink with Alginate

Formulation 1: AL-HG-1

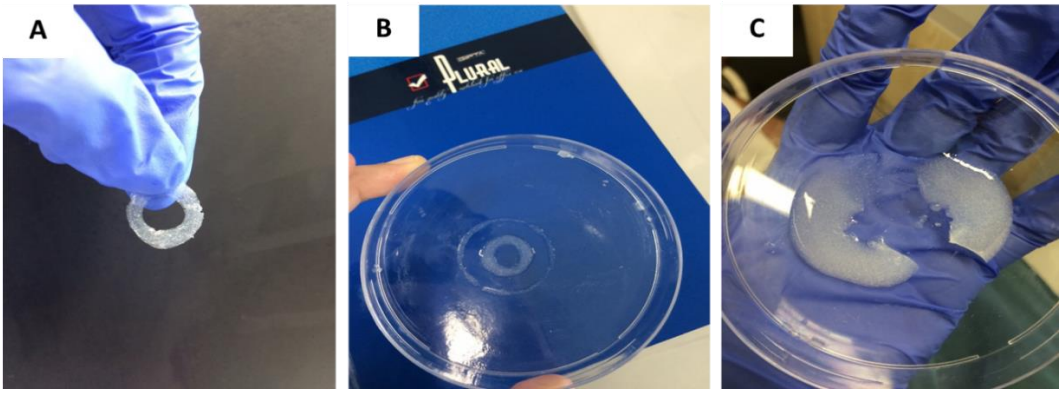

Figure 4.20 3D printing of AL-HG-1: Alginic acid (4% w/v) + PEGdiMA (20% v/v) + Irgacure (1,5% w/v).

Formulation 2: AL-HG-2

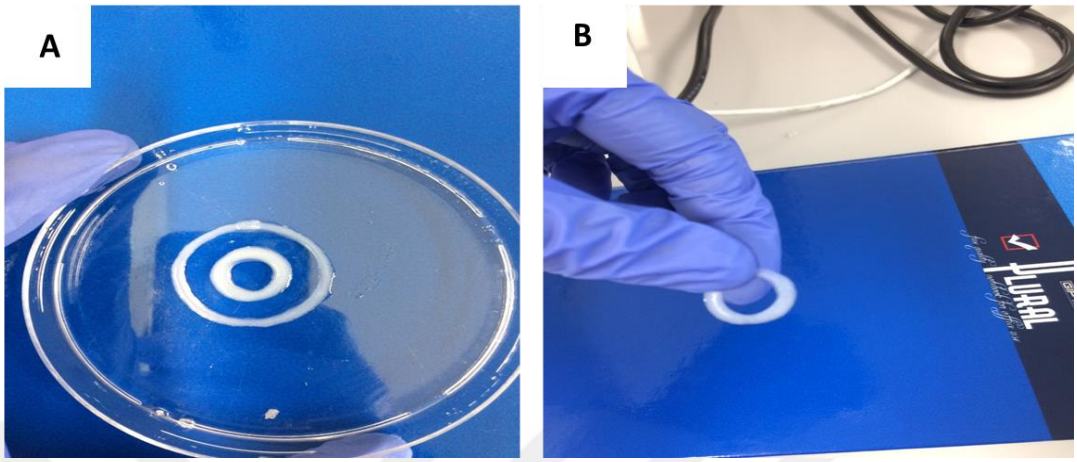

Figure 4.21 3D printing of AL-HG-2: Alginic acid (4% w/v) + PEGdiMA (10% v/v)+ Irgacure (1,8% w/v).

4.4 Characterization of Hydrogels

Biomaterial inks were evaluated by swelling capacity, rheological properties, functional group analysis, morphological analysis and methacrylated natural polymers were evaluated by the measurement of methacrylation ratio.

Swelling Test

The determination of water uptake of hydrogels was evaluated as a swelling test. Lyophilized and dried hydrogels were incubated in water at room temperature. Hydrogels were weighed by taking from the beaker at certain time intervals.

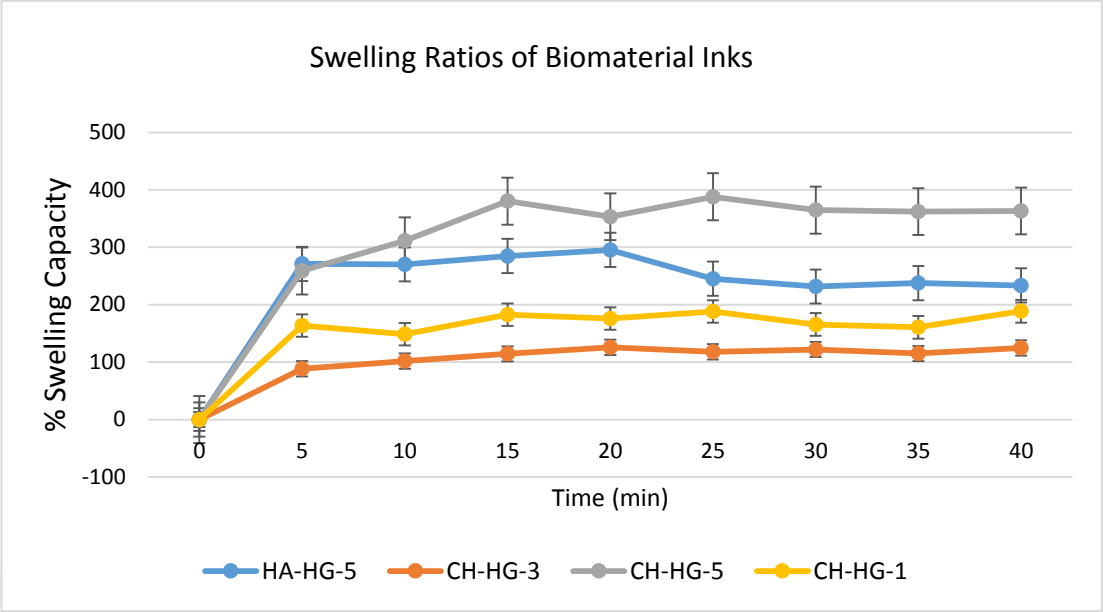

Figure 4.22 Swelling Behaviors of Biomaterial inks.

Rheological Evaluation

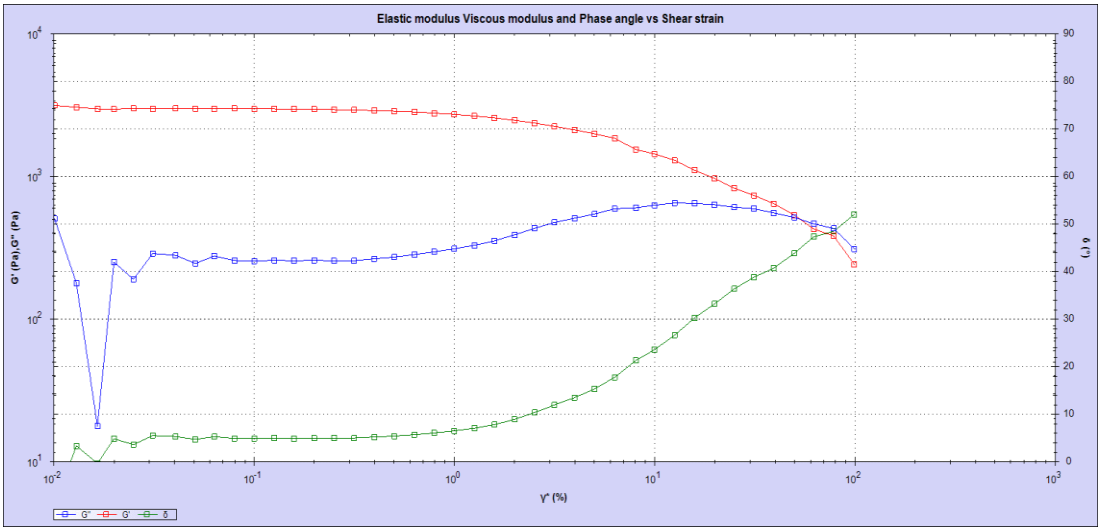

Figure 4.23 Rheological Evaluation for HA-HG-5. G' and G''values between 10^1 and 10^4 vs shear strain (γ) between 10^{-2} and 10^2 at 1 Hz frequency and 25 ºC.

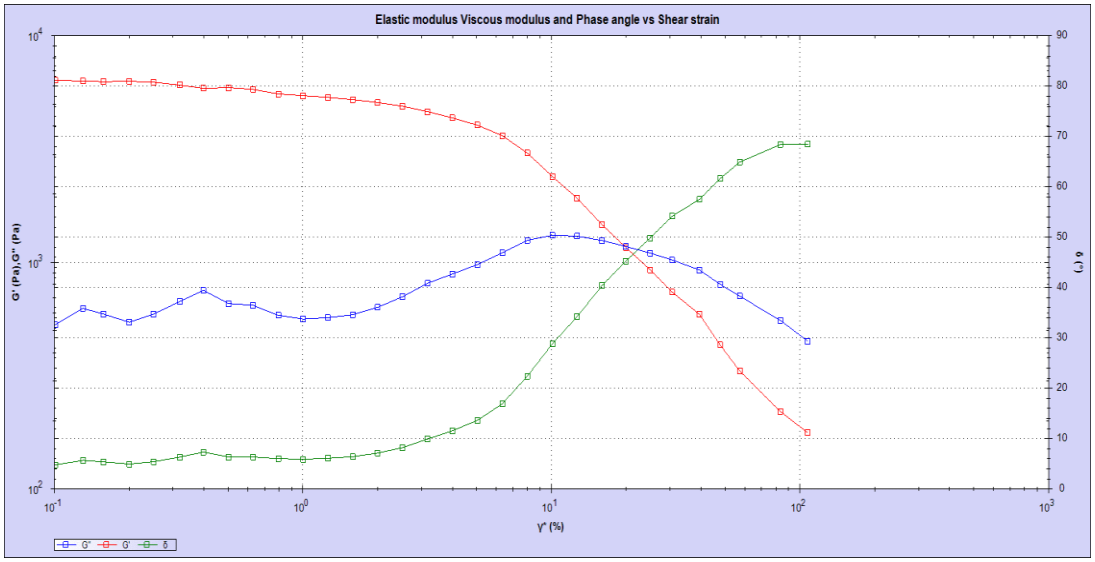

Figure 4.24 Rheological Evaluation for CH-HG-5. G' and G''values between 10^2 and 10^4 vs shear strain (γ) between 10^{-1} and 10^2 at 1 Hz frequency and 25 ºC.

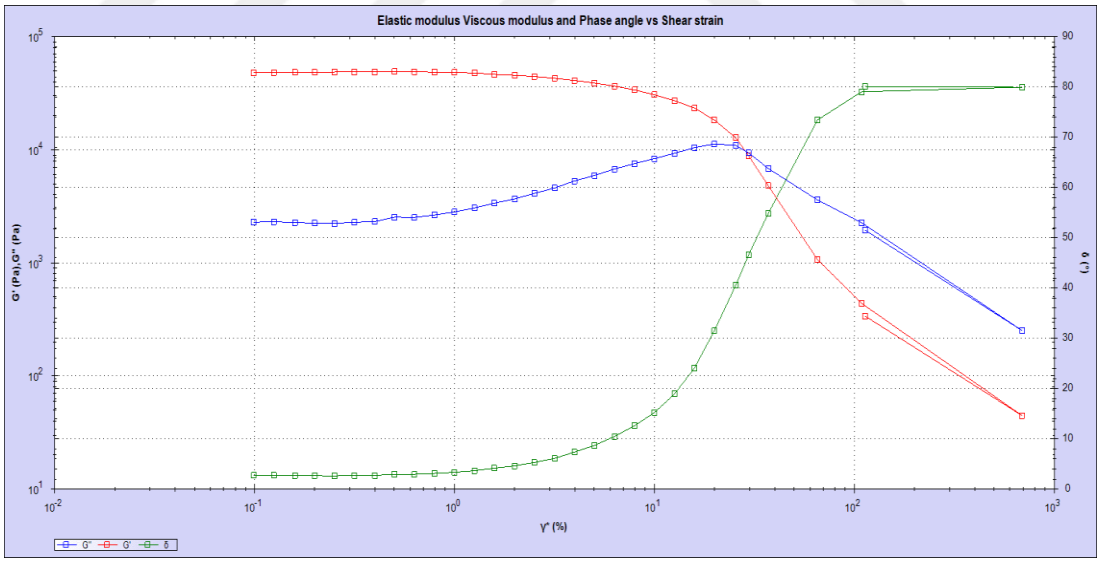

Figure 4.25 Rheological Evaluation for CH-HG-1. G' and G''values between 10^1 and 10^5 vs shear strain (γ) between 10^{-1} and 10^3 at 1 Hz frequency and 25 ºC.

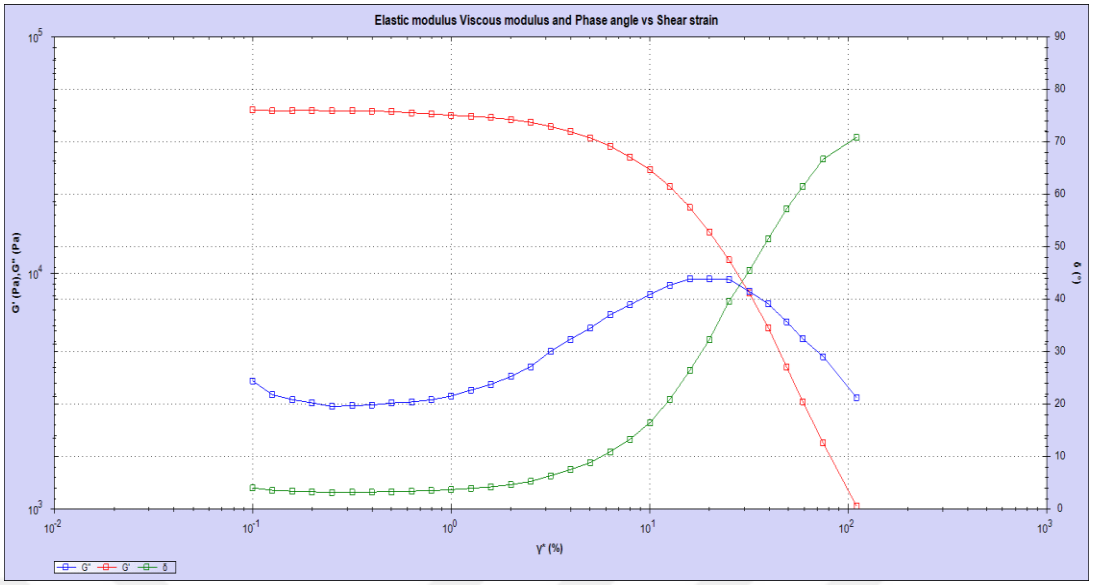

Figure 4.26 Rheological Evaluation for CH-HG-3. G' and G''values between 10^3 and 10^5 vs shear strain (γ) between 10^{-1} and 10^2 at 1 Hz frequency and 25 ºC.

Functional Group Analysis (FT-IR)

Functional group analysis of 4 biomaterial ink formulations:

HA-HG-5; (2,5 % (w/v) Hyaluronic Acid + 10 % (w/v) Gel-MA+ 25 % (v/v) PEGdiMA),

CH-HG-5; (2 % (w/v) Chitosan + 10 % (w/v) Gel-MA+ 25 % (v/v) PEGdiMA),

CH-HG-1; (2,5 % (w/v) Chitosan + 25 % (v/v) PEGdiMA),

CH-HG-3; (2 % (w/v) Chitosan + 2 % (w/v) Gelatin + 25 % (v/v) PEGdiMA).

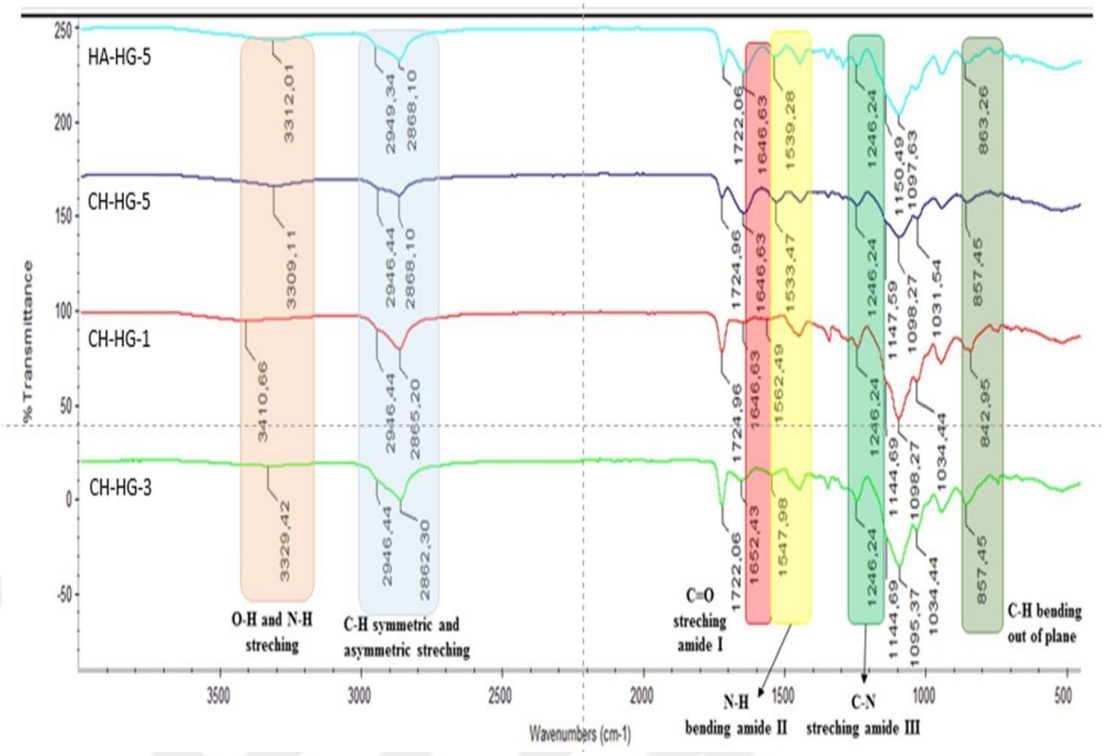

Figure 4.27 FT-IR spectra of Biomaterial Ink Formulations.

Morphological analysis (Scanning Electron Microscopy-SEM)

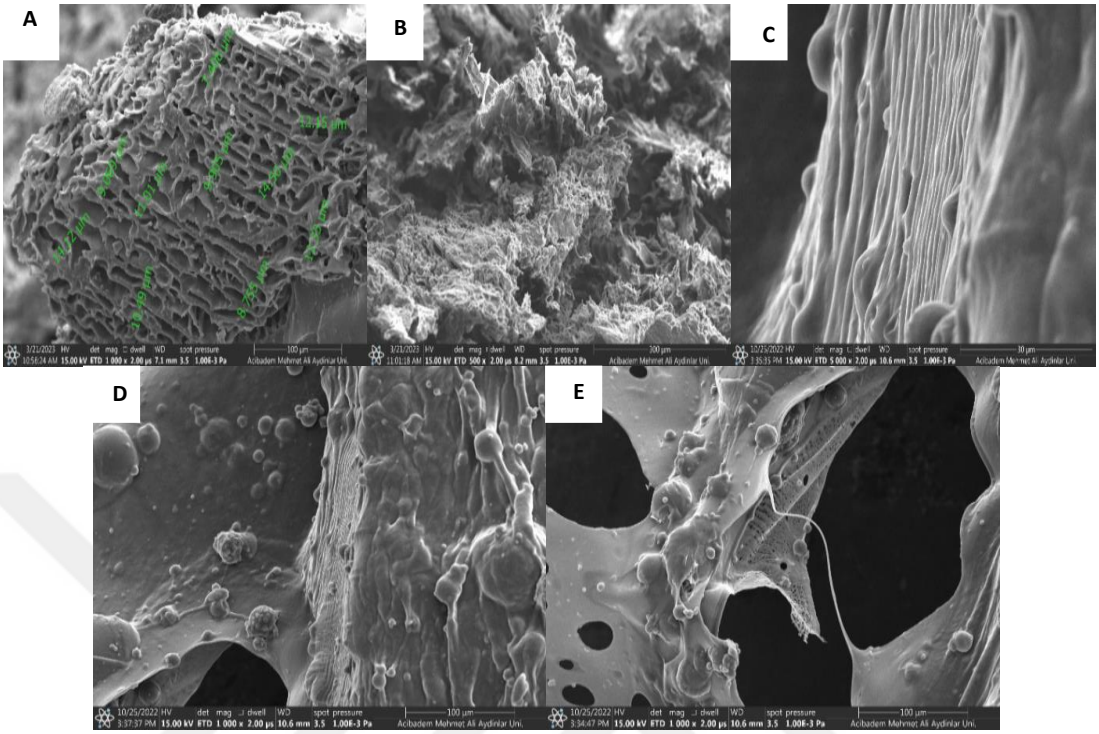

Figure 4.28 SEM images of HA-HG-5: (2,5 % (w/v) Hyaluronic Acid + 10 % (w/v) Gel-MA+ 25 % (v/v) PEGdiMA) Scale bar: (A) 100 µm, (B) 300 µm, (C) 30 µm, (D) 100 µm, (E) 100 µm.

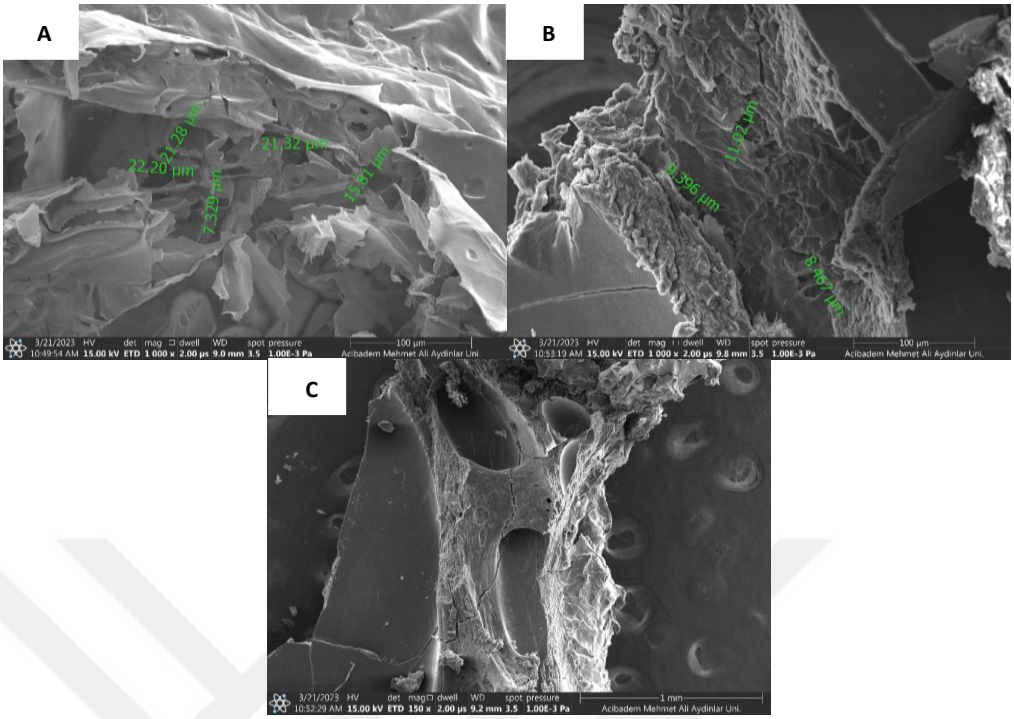

Figure 4.29 SEM images of CH-HG-5: (2 % (w/v) Chitosan + 10 % (w/v) Gel-MA+ 25 % (v/v) PEGdiMA) Scale bar: (A) 100 µm, (B) 100 µm, (C) 1mm.

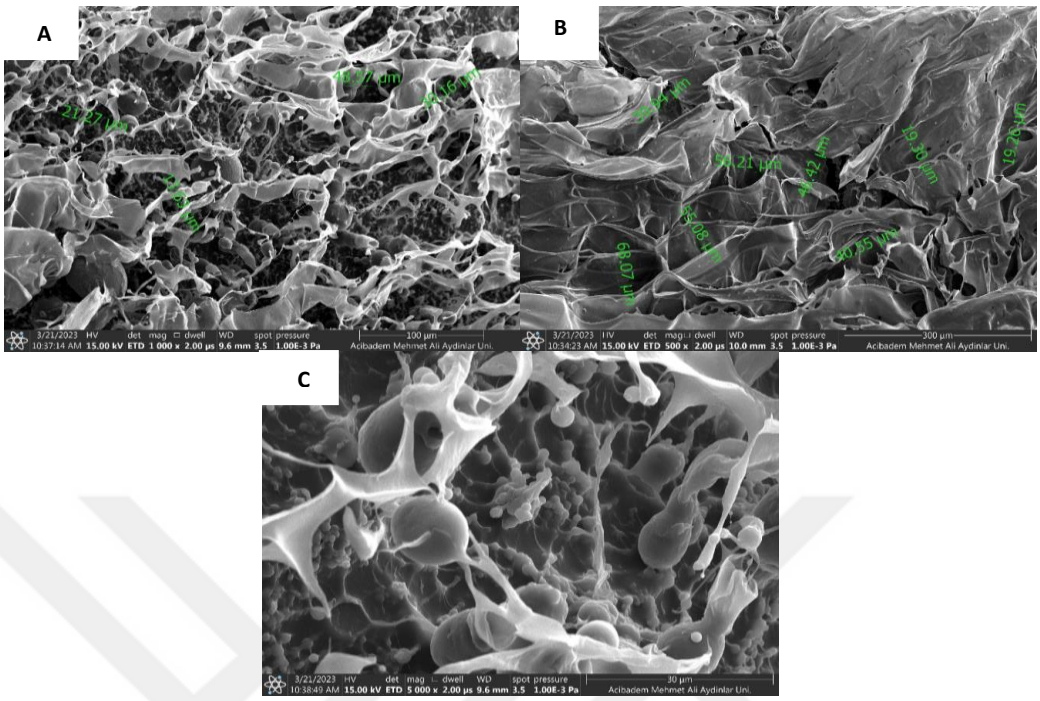

Figure 4.30 SEM images of CH-HG-1: (2,5 % (w/v) Chitosan + 25 % (v/v) PEGdiMA) Scale bar: (A) 100 µm, (B) 300 µm, (C) 30 µm.

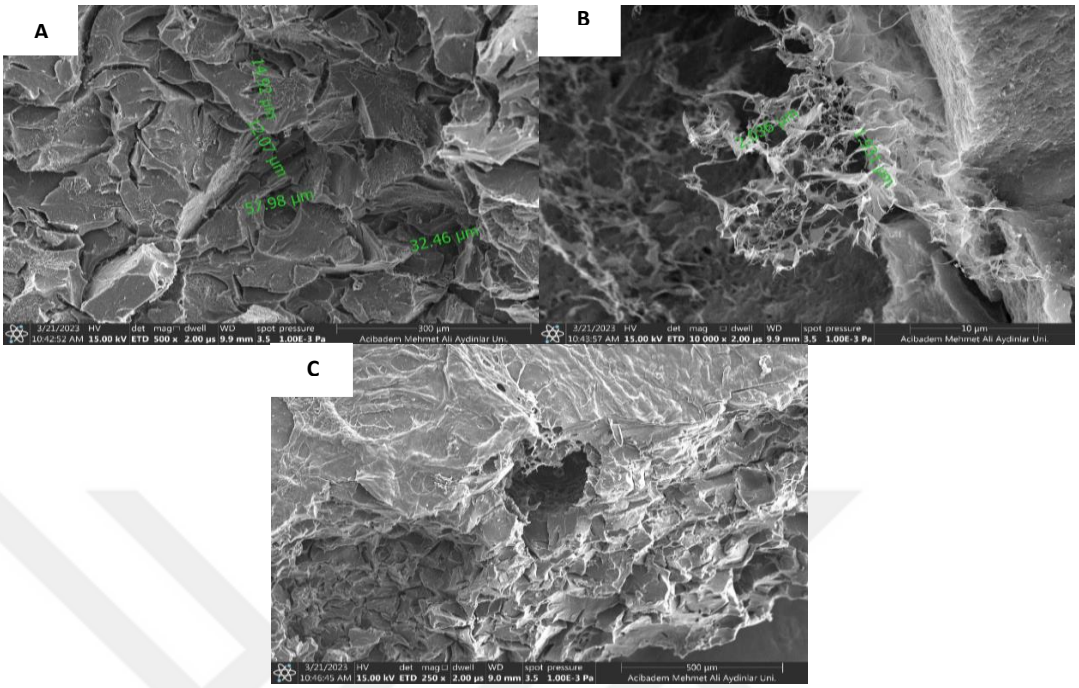

Figure 4.31 SEM images of CH-HG-3: (2 % (w/v) Chitosan + 2 % (w/v) Gelatin + 25 % (v/v) PEGdiMA) Scale bar: (A) 300 µm, (B) 10 µm, (C) 500 µm.

Gel Conversion of Hydrogels

Table 4.1 Gel Conversion Rates of Hydrogels.

Hydrogel Name	**Gel Yield (%)**
Gel-MA	76
Ha-MA	100
Al-MA	100
Chi-MA	69
Hydrogel Name	**Gel Conversion (%)**
HA-HG-5: 2,5% (w/v) Hyaluronic Acid + 10% (w/v) Gel-MA + 25% (v/v) PEGdiMA	96
CH-HG-5: 2% (w/v) Chitosan + 10% (w/v) Gel-MA + 25% (v/v) PEGdiMA	97
CH-HG-1: 2,5% (w/v) Chitosan + 25% (v/v) PEGdiMA	96
CH-HG-3: 2% (w/v) Chitosan + 2% (w/v) Gelatin + 25% (v/v) PEGdiMA	95

Methacrylation Ratio of Methacrylated Natural Polymers

Standard Table Report

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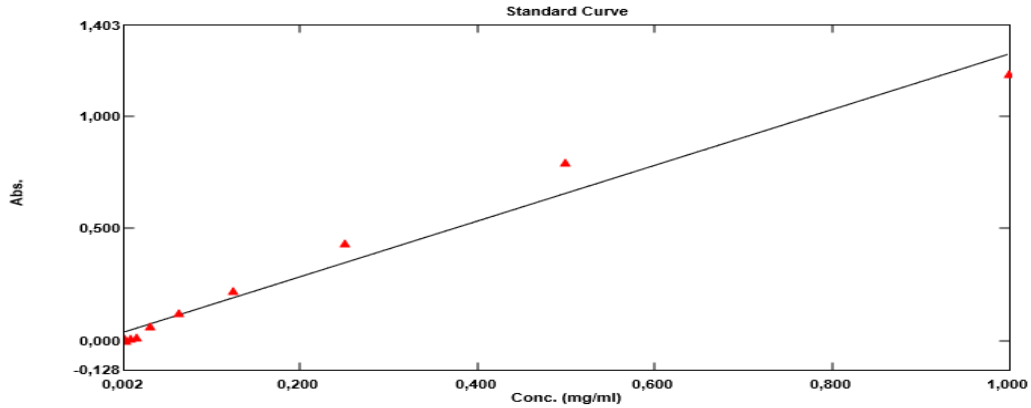
File Name: C:\Users\Spektroskopi.ACU\Desktop\Emine\PEGMA Photometric 281221.pho



y = 1,23894 x + 0,0377815
r2 = 0.97196

Standard Table

	Sample ID	Type	Ex	Conc	WL234,0	Wgt.Factor	Comments
1	10PEGMA	Standard		0.002	0.007	1.000	
2	9PEGMA	Standard		0.004	-0.000	1.000	
3	8PEGMA	Standard		0.008	0.008	1.000	
4	7PEGMA	Standard		0.015	0.014	1.000	
5	6PEGMA	Standard		0.031	0.064	1.000	
6	5PEGMA	Standard		0.063	0.126	1.000	
7	4PEGMA	Standard		0.125	0.224	1.000	
8	3PEGMA	Standard		0.250	0.433	1.000	
9	2PEGMA	Standard		0.500	0.792	1.000	
10	1PEGMA	Standard		1.000	1.185	1.000	
11							

Figure 4.32 Standard Curve of PEGMEMA at 234 nm by using UV-Vis at Photometric Mode.

Table 4.2. Methacrylation Ratio of Methacrylated Natural Polymers: Gel-MA, Ha-MA, Al-MA and Chi-MA measured by UV-Vis Spectrophotometer.

Hydrogel Name	Methacrylation Ratio (mg/mL)
Gel-MA	21
Ha-MA	22
Al-MA	18
Chi-MA	9,2

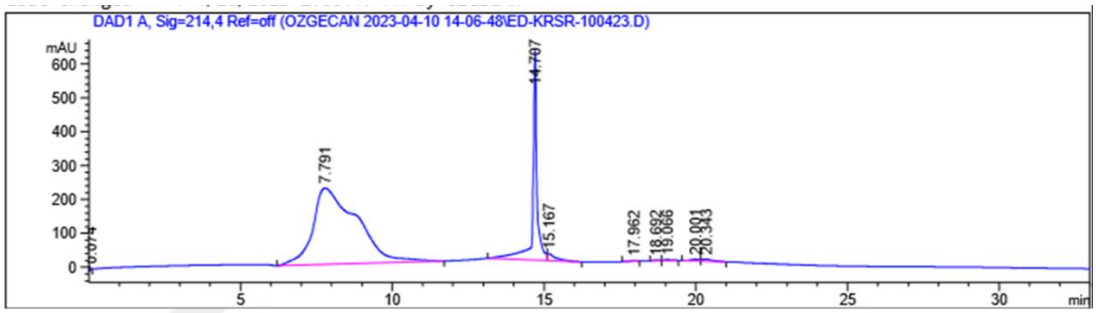
Table 4.3. Optimization of Hydrogels.

Sample Name	Components	UV cross-linkability	3D-printability
HA-HG-1	Hyaluronic acid (4 % w/v)+PEGdiMA (10 % v/v)+Irgacure (0,9 % w/v)	+	-
HA-HG-2	Hyaluronic acid (2 % w/v)+PEGdiMA (25% v/v)+Irgacure (1 % w/v)	-	-
HA-HG-3	Hyaluronic acid (3 % w/v)+PEGdiMA (20 % v/v)+Irgacure (1 % w/v)	+	-
HA-HG-4	Hyaluronic acid (2,5 % w/v)+Gel-MA (5 % w/v)+PEGdiMA (25 % v/v)+Irgacure (1 % w/v)	+	+
HA-HG-5	Hyaluronic acid (2,5 % w/v)+Gel-MA (10 % w/v)+PEGdiMA (25 % v/v)+Irgacure (2 % w/v)	+	+
HA-HG-6	Hyaluronic acid (2 % w/v)+Al-MA (2% w/v)+PEGdiMA (30 % v/v)+Irgacure (2 % w/v)	+	-
CH-HG-1	Chitosan (2,5 % w/v)+PEGdiMA (25 % v/v)+Irgacure (2 % w/v)	+	+
CH-HG-2	Chitosan (1,5 % w/v)+Gel-MA (3 % w/v)+PEGdiMA (25 % v/v)+Irgacure (2 % w/v)	+	-
CH-HG-3	Chitosan (2 % w/v)+Gelatin (2 % w/v)+PEGdiMA (25 % v/v)+Irgacure (2 % w/v)	+	+
CH-HG-4	Chitosan (1,5 % w/v)+Gelatin (10 % w/v)+PEGdiMA (25 % v/v)+Irgacure (2 % w/v)	+	-
CH-HG-5	Chitosan (2 % w/v)+Gel-MA (10 % w/v)+PEGdiMA (25 % v/v)+Irgacure (2 % w/v)	+	-
CH-HG-6	Chi-MA (2,5 % w/v)+Gelatin (10 % w/v)+PEGdiMA (25 % v/v)+Irgacure (2 % w/v)	+	-
AL-HG-1	Alginic acid (4 % w/v)+PEGdiMA (20 % v/v)+Irgacure (1,5 % w/v)	+	-
AL-HG-2	Alginic acid (4 % w/v)+PEGdiMA (10 % v/v)+Irgacure (1,8 % w/v)	+	-

4.5 Characterization of KRSR Peptide

High Pressure Liquid Chromatography (HPLC)

KRSR peptide was characterized by using High Pressure Liquid Chromatography (HPLC).



Signal 1: DAD1 A, Sig=214,4 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	0.074	BB	0.0488	13.63200	4.56538	0.0466
2	7.791	BB	1.3483	2.26039e4	224.57132	77.2043
3	14.707	BV	0.1269	5889.32471	619.81091	20.1152
4	15.167	VB	0.2751	417.03958	20.17793	1.4244
5	17.962	BB	0.1920	29.48879	2.45784	0.1007
6	18.692	BV	0.1971	34.46313	2.84978	0.1177
7	19.066	VB	0.2511	46.74160	2.71184	0.1596
8	20.001	BV	0.2824	85.49877	4.52520	0.2920
9	20.343	VB	0.3322	157.93156	6.71979	0.5394

Figure 4.33 HPLC result of KRSR peptide.

4.6 *In-vitro* Cytotoxicity Assay

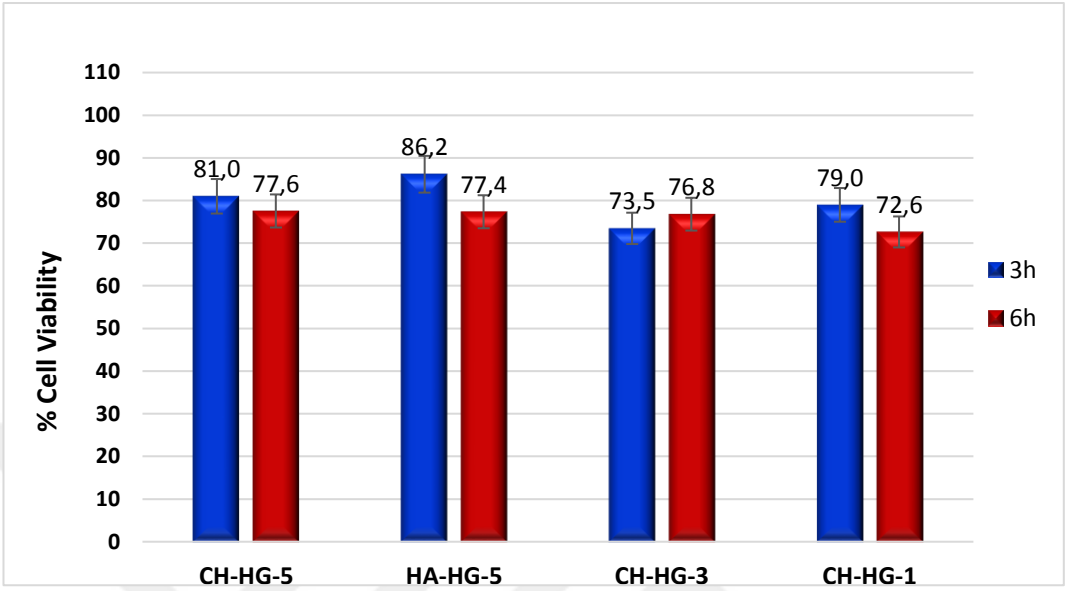


Figure 4.34 *in-vitro* cytotoxicity results of biomaterial inks on the L929 mouse fibroblast cell line: CH-HG-5, HA-HG-5, CH-HG-3 and CH-HG-1 at 3h and 6h incubation time.

4.7 Preparation of with and without Peptide Encapsulated Biomaterial Inks

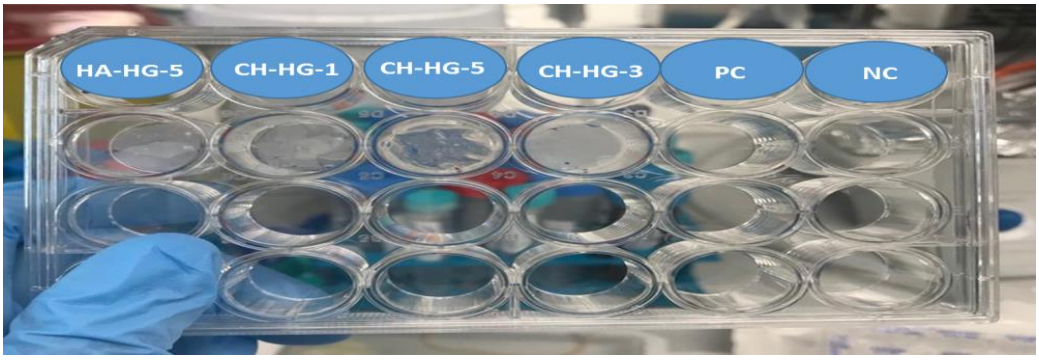


Figure 4.35 Biomaterial inks without KRSR peptide prepared in 0,2 mm circular disk for 4 hydrogels; HA-HG-5, CH-HG-5, CH-HG-3 and CH-HG-1.

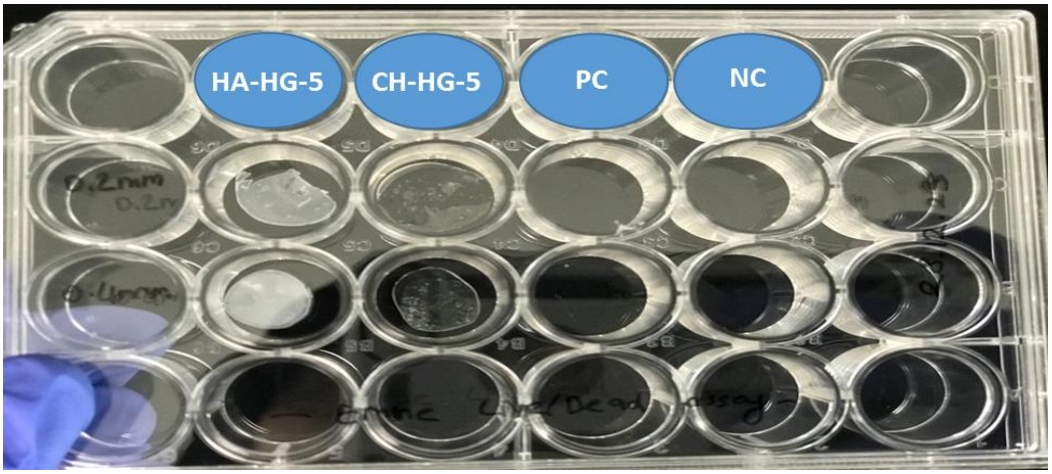


Figure 4.36 KRSR encapsulated biomaterial inks prepared in 0,2 mm circular disk for 2 hydrogels; HA-HG-5 and CH-HG-5.

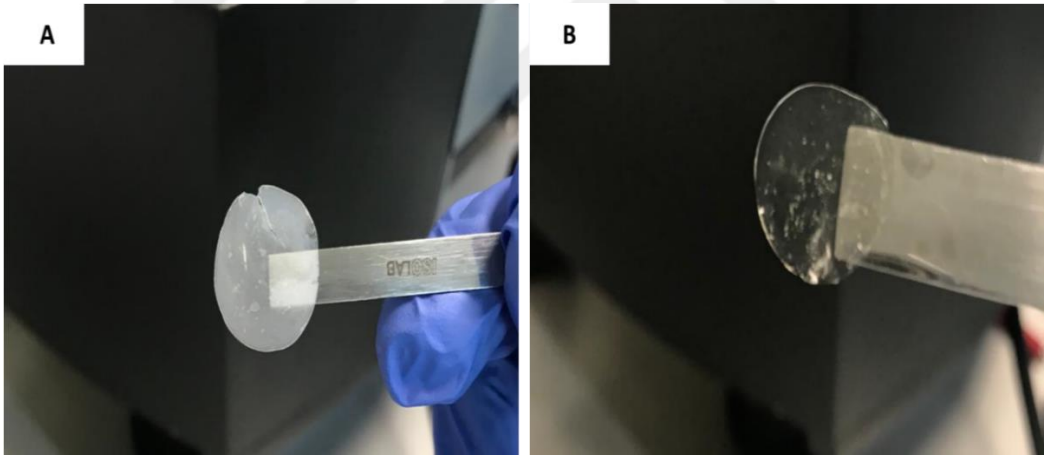


Figure 4.37 KRSR encapsulated HA-HG-5 prepared in 0,2 mm circular disk (A). KRSR encapsulated CH-HG-5 prepared in 0,2 mm circular disk (B).

4.8 Cell Adhesion Test

Cell adhesion tests were examined for 4 biomaterial ink formulations without KRSR peptide which are HA-HG-5, CH-HG-5, CH-HG-3 and CH-HG-1.

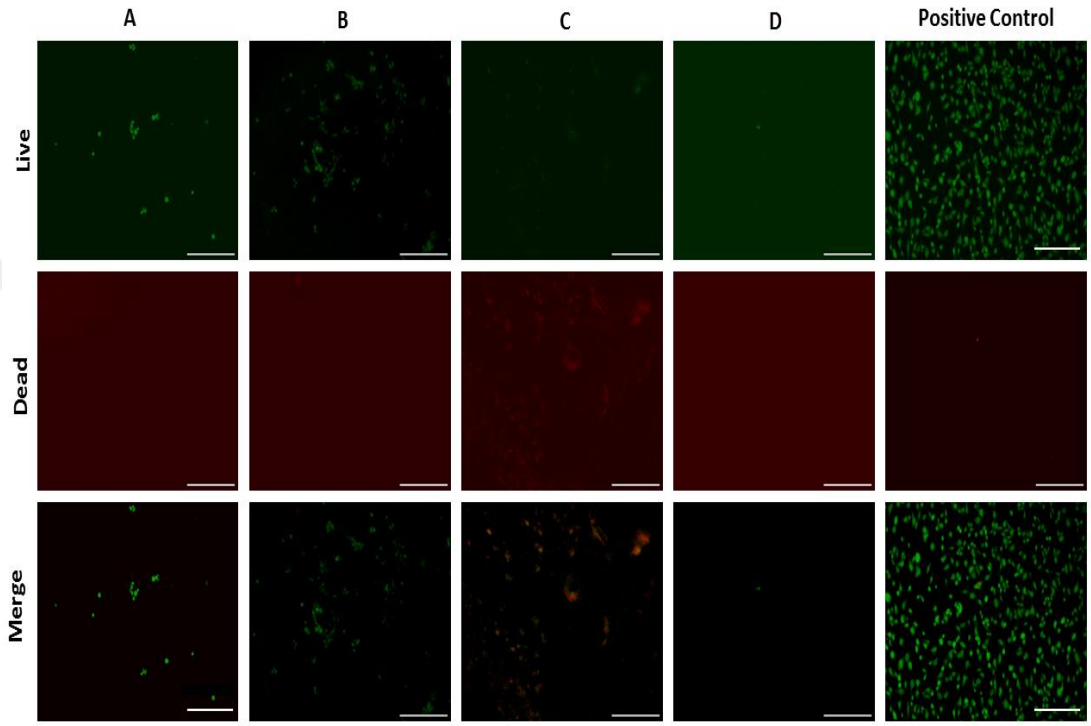


Figure 4.38 Live-dead assay's images of 4 biomaterial ink formulations for cell adhesion of hFOB 1.19 in hydrogels without KRSR Peptide on 1 day. (scale bar: 200 μm) hFOB 1.19 in HA-HG-5 (A). hFOB 1.19 in CH-HG-5 (B). hFOB 1.19 in CH-HG-1 (C). hFOB 1.19 in CH-HG-3 (D).

Cell adhesion tests were examined for KRSR peptide encapsulated 2 biomaterial ink formulations which are HA-HG-5 and CH-HG-5.

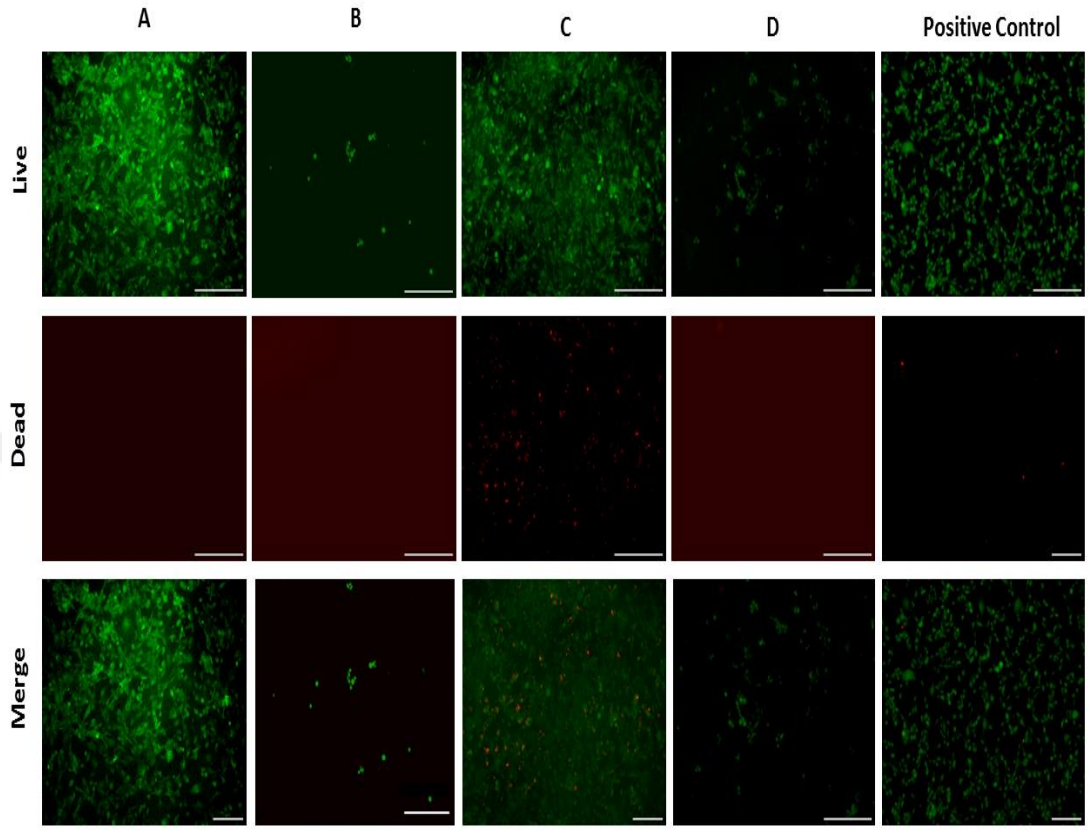


Figure 4.39 Live-dead assay's images of 2 biomaterial ink formulations for cell adhesion of hFOB 1.19 in hydrogels with and without KRSR Peptide on 1 day. (scale bar: 200 μm) hFOB 1.19 in HA-HG-5 with KRSR (A). hFOB 1.19 in HA-HG-5 without KRSR (B). hFOB 1.19 in CH-HG-5 with KRSR (C). hFOB 1.19 in CH-HG-5 without KRSR (D).

4.9 Alizarin Red S Staining

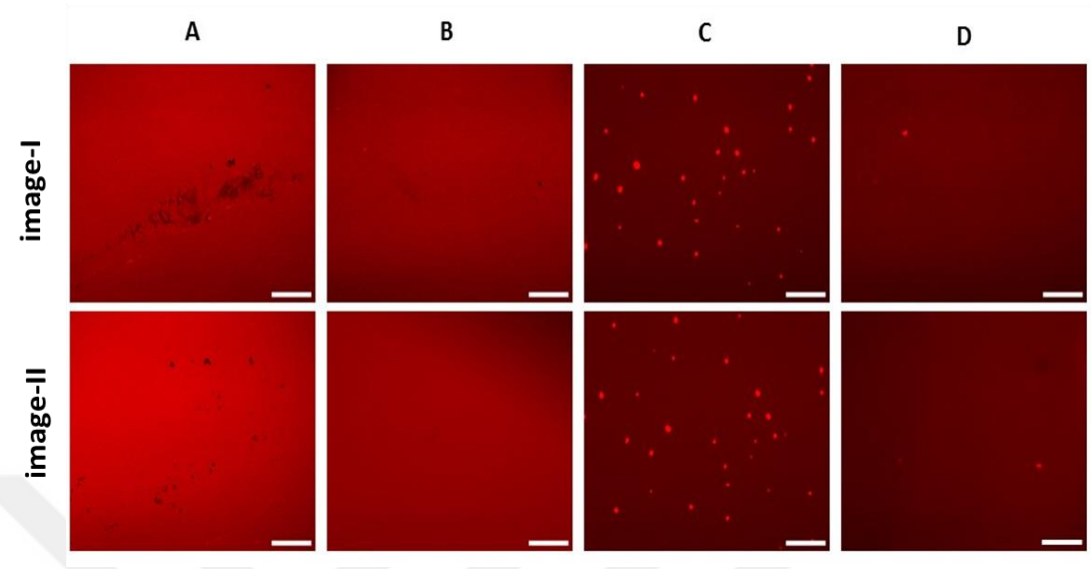


Figure 4.40 Effect of KRSR peptide for mineralization was evaluated by Alizarin Red S Staining. Staining was performed for HA-HG-5 with and without KRSR peptide (scale bar: 200 μm). Mineralization of hFOB 1.19 in the presence of PBS in HA-HG-5 with KRSR (A). Mineralization of hFOB 1.19 in the presence of PBS in HA-HG-5 without KRSR (B). Mineralization of hFOB 1.19 in the presence of PBS in 2D-well plate (C). Mineralization of hFOB 1.19 in the absence of PBS in 2D-well plate (D).

5. DISCUSSION

Within the framework of this study, KRSR peptide encapsulated and 3D printable hydrogels as polymeric biomaterial inks were designed for bone tissue engineering and evaluated with *in-vitro* studies. Thanks to this project, it will be possible to print 3D hydrogels from biocompatible, biodegradable, non-toxic, cost-effective polymers that are suitable for patients who suffer from bone-related diseases. Here, non-functionalized natural polymers such as hyaluronic acid, chitosan and alginate were used due to their suitable viscosity while gelatin was used to increase cell adhesion into the hydrogels. Methacrylic anhydride functionalized natural polymers were synthesized to crosslink composite hydrogels. Moreover, Poly(ethylene glycol)di Methacrylate (PEGdiMA) is a synthetic polymer for gives mechanical strength and increases the crosslinkability of hydrogels.

Before starting to make biomaterial inks, components of biomaterial inks were synthesized and optimized. Methacrylic anhydrite (MA) was conjugated into gelatin (Gel-MA), chitosan (Chi-MA), alginate (Al-MA) and hyaluronic acid (Ha-MA). After synthesizing, pre-polymers were lyophilized for characterization. First of all, a UV-Vis Spectrophotometer (Shimadzu UV-VIS 2600) was used to see how much Methacrylic anhydrite (MA) was bound to the side chains of the natural polymers and the absorbance of MA was measured using the PEGMEMA calibration curve which contains a single methacrylate in its structure and whose chain part consists of ethylene glycol molecules that do not absorb at 234 nm where the methacrylate group gives maximum absorption. For each pre-polymer, the required Polymer-MA conjugation values for cross-linking were found at 234 nm as seen in Table 4.2. It was then crosslinked in the presence of UV light (365 nm) to see how much Irgacure (photoinitiator) could form a hydrogel.

After that, the gel conversion rate (%) of these hydrogels was measured as seen in Table 4.1 (Gel-MA, Ha-MA, Al-MA and Chi-MA). Biomaterial ink formulations were prepared as triple combinations by using non-functionalized natural polymers, methacrylic anhydride conjugated natural polymers and PEGdiMA. Hyaluronic acid, Alginate and Chitosan were used due to their viscosities. Especially gelatin or Gel-MA was used in each formulation as it contains RGD which provides cell adhesion to the hydrogels. On the other hand, PEGdiMA supported and increased the crosslinking speed and mechanical properties of polymers in the presence of Irgacure. All triple combinations were tried respectively starting from hyaluronic acid, chitosan and alginate by using (extrusion-based bioprinting) Axolotl Bioprinter Dual Print Head System (Axolotbio, Türkiye) as seen in 4.3 section. HA-HG-1, HA-HG-2, HA-HG-3, HA-HG-4 and HA-HG-6 were eliminated because they could not be 3D-printed as seen 4.3 section except HA-HG-5 as seen in Figure 4.12. HA-HG-5 was 3D-printed and crosslinked as 30 layers successfully.

For combinations with chitosan, CH-HG-1, CH-HG-2, CH-HG-3, CH-HG-4, CH-HG-5, CH-HG-6 were tried and CH-HG-1, CH-HG-3 were chosen as 3D printable almost 5 layers. Although CH-HG-5 was not printable as seen in Figure 4.18, it was chosen as a control to demonstrate cell adhesion effect of the chitosan since it's positively charged in cell adhesion tests. For combinations with alginate, AL-HG-1 and AL-HG-2 were tried. However, they were eliminated because couldn't be 3D printable.

A swelling test was made to measure the water uptake capacity of hydrogel scaffolds. After the lyophilization process, dried hydrogel pieces were weighed and put into distilled water to swell. Swollen hydrogels were weighted and noted at a certain time interval every 5 minutes as seen in Figure 4.22.

In Figure 4.22, the graphic shows the changing of weights of hydrogel scaffolds as uptake the water. For CH-HG-5 and CH-HG-1, their weights have increased until the 25^{th} minute. After that, they reached maximum capacity and started to decrease because of disruption. On the other hand, weights of CH-HG-3 and HA-HG-5 have increased until the 20^{th} minute. After this point, swelling capacities have reached maximum level and started to decrease.

Rheological behaviors of hydrogel scaffolds were examined by using a rheometer (Malvern, Kinexus). Here, oscillation test was maintained to examine the deformation rate of prepared hydrogels at 25 ºC (constant), 1 Hz frequency (constant) and in a 1 mm gap by using J2 SR 4703 SS geometry. G″ represents viscosity (loss modulus) and G′ represents elasticity (storage modulus). G' and G'' amplitudes are independent between 0,01 (%) and 10 (%) strain values for 4 hydrogels means that Linear viscoelastic region (LVR) is present in these intervals. After a while (between 10 and 100 % strain), G' and G'' overlap and split. The overlap point is where the deformation in hydrogel structure happens, and the degradation starts. After this point, while the value of G'decreases, the value of G'' increases relatively more than G' which means the liquid-like properties of hydrogels increase.

As seen in Figures 4.23, 4.24, 4.25 and 4.26, deformation occurs at different points for all 4 hydrogels. This is because different biomaterials may have different effects on stress. When 4 hydrogels were compared, it's seen that biomaterial inks containing chitosan CH-HG-1, CH-HG-2 and CH-HG-5 have higher G' values than HA-HG-5 which represent the solid-like nature of the biomaterials as expected. Because the mechanical strength of hyaluronic acid is weaker than chitosan. The other possibility is Gel-MA is negatively charged and chitosan is positively charged which can cause a physical bonding in the biomaterial ink while hyaluronic acid is negatively charged.

After cross-linking, biomaterial inks were lyophilized and dried for FT-IR analysis (in Figure-4.27). 4 biomaterial inks include O-H and C-H stretching around 3300-3500 cm^{-1} which is characteristic of polysaccharide-derived polymers. Also, it's seen carbonyl peaks C=O stretching amide I around 1640-1650 cm^{-1} belonging to carboxylic acid groups. C-H symmetric and asymmetric stretching in the structures of the hydrogels are represented around 2800-2950 cm^{-1}. On the other hand, it's seen the peaks of C-O-C bonds of PEG chain in the PEGdiMA structure around 1100-1140 cm^{-1}.

The morphological structure of hydrogels was analyzed via Scanning Electron Microscopy-SEM (THERMO-FISHER SCIENTIFIC QUATTRO S ESEM). In Figure 4.28 which belongs to HA-HG-5 demonstrates the porous (A) and smooth surface (C) and (D). Moreover, It's clearly seen that 30 layers of HA-HG-5 in the figure.4.27 (C) and (D) while (E) shows crosslinking points of HA-HG-5. Figures 4.29, 4.30 and 4.31 demonstrates respectively, CH-HG-5, CH-HG-1 and CH-HG-3 hydrogels. It's seen that porous structures of hydrogels.

The gel conversion rate was calculated by how much viscous gel was converted to crosslinked gel after drying. It was observed both crosslinked methacrylated hydrogels and biomaterial bioink hydrogels. All of them have above 90 % gel-conversion rate except Gel-MA and Chi-MA as seen in Table 4.1.

Another characterization method is measurement of methacrylation ratio of methacrylated natural polymers. This characterization method was performed to determine how much methacrylate groups are attached to the side chains of the methacrylated natural polymers. The degree of methacrylation was measured quantitatively using a UltraViole-Visible (UV-Vis) spectrophotometer (Shimadzu UV-VIS 2600). A calibration curve was prepared for the methacrylate group using Poly(ethylene glycol) monomethyl ether methacrylate (PEGMEMA) monomer, which contains a single methacrylate in its structure and whose chain part consists of ethylene glycol molecules that do not absorb at 234 nm, where the methacrylate group gives maximum absorption as seen in Table 4.2. By using this calibration curve to be prepared at 234 nm, the methacrylation degrees of the polymers to which methacrylate was added to their structure were calculated as their weight ratio to the polymer as seen in Figure 4.32. These ratios were enough to crosslink methacrylated natural polymers.

After synthesizing of KRSR peptide, High Pressure Liquid Chromatography-HPLC (260 Infinity Quaternary LC systems, Agilent Technologies) was observed to measure purification of peptide. It’s seen that retention time of the peak is almost 4 mins which is greatest area in the chromatogram (77 %) at 214 nm which belongs peptide absorbance value as seen in Figure 4.33.

Since toxicity is one of the major problems for biomaterials for tissue engineering, biomaterial ink scaffolds were evaluated for *in-vitro* cytotoxicity by using CCK-8 cell counting assay against the L929 mouse fibroblast cell line with indirect method. In Figure 4.34, it's seen that our hydrogel scaffolds' viabilities are above 70 % means non-toxic according to indicated rules by the FDA at both 3h and 6h incubation time. While HA-HG-5 has highest viability ratio, CH-HG-3 has lowest ratio at 3h incubation time. If we look at the 6h incubation time, it's seen that CH-HG-5 has highest viability ratio while CH-HG-1 has lowest one.

Cell adhesion test was run for another major part of the *in-vitro* evaluation of HA-HG-5, CH-HG-5, CH-HG-3 and CH-HG-1 to demonstrate effect of the cell adhesion peptide found in biomaterial ink combinations on the hFOB 1.19 cell line. Firstly, hydrogel scaffolds without KRSR peptide were prepared in 0,2 mm circular disk via Rheometer (Malvern, Kinexus) and OmniCure Series 2000 (for crosslinking) as seen in Figure 4.35. After that, Live/Dead Cell Viability Assay Kit (ab287858) was applied and visualized with fluorescence microscope (EVOS^{TM} M5000 Cell Imaging System, ThermoFisher) for 4 hydrogel scaffolds. The results demonstrate that CH-HG-5 has highest cell adhesion compared to other hydrogel scaffolds means that Gel-MA and chitosan together increased hFOB 1.19 cell adhesion in Figure 4.38 (B). On the other hand, HA-HG-5 which includes hyaluronic acid and Gel-MA has cell adhesion effect relatively less than CH-HG-5 in Figure 4.38 (A). However, CH-HG-1 which doesn't include Gel-MA or gelatin and CH-HG-3 which has gelatin polymer have no appreciable cell adhesion as seen in 4.38 (C) and (D). It is clear from here, methacrylated gelatin has more cell adhesive effect than non-functionalized gelatin due to RGD peptide with chitosan and hyaluronic acid. In this framework, CH-HG-1 and CH-HG-3 were eliminated because they didn't have cell adhesion effects although they are 3D printable.

KRSR peptide encapsulated HA-HG-5 and CH-HG-5 were prepared in 0,2 mm circular disk to demonstrate cell adhesion effect of KRSR peptide. The results clearly show that peptide-encapsulated hydrogels increased hFOB 1.19 cell adhesion compared to hydrogels without KRSR peptide as seen in 4.39. When HA-HG-5 with KRSR in Figure 4.39 (A) and CH-HG-5 with KRSR in Figure 4.39 (B) are compared, HA-HG-5 is more suitable than CH-HG-5 in terms of cell differentiation in morphology and proliferation because there are no dead cells in HA-HG-5 while CH-HG-5 does have in Figure 4.39 (C). This may be because CH-HG-5 promotes uncontrolled hFOB 1.19 cell proliferation thus increasing cell death. Consequently, CH-HG-5 was eliminated from mineralization tests for the reasons mentioned. Mineralization test was run for HA-HG-5. It is clearly seen that KRSR peptide increased the calcification of hFOB 1.19 cells after PBS treatment in Figure 4.40 (A) compared to HA-HG-5 without KRSR peptide in Figure 4.40 (B).

6. CONCLUSION

First of all, methacrylated natural polymers were synthesized to crosslink biomaterial inks. Moreover PEGdiMA (750 Mn) was used to increase mechanical strength of hydrogel scaffolds and decrease crosslinking duration of biomaterial inks during 3D printing. Alginate (low viscosity), chitosan (high molecular weight) and hyaluronic acid (high molecular weight) were used in the triple combinations to increase the viscosity of biomaterial inks to be able to 3D printable. Irgacure was used as photoinitiator in appropriate amounts. HA-HG-5, CH-HG-5, CH-HG-3 and CH-HG-1 were chosen among biomaterial ink combinations since they are 3D printable except CH-HG-5. It was chosen to observe cell adhesive feature of the chitosan-Gel-MA combination.

After that, the cytotoxicity test which is first step of the *in-vitro* evaluation was applied against L929 cell line for HA-HG-5, CH-HG-5, CH-HG-3 and CH-HG-1. Here, HA-HG-5 has highest viability ratio while CH-HG-3 has lowest ratio at 3h incubation time. If we look at the 6h incubation time, it's seen that CH-HG-5 has highest viability ratio while CH-HG-1 has lowest one. The second step was cell adhesion test for these four biomaterial ink combinations. The test was run for these hydrogels without KRSR peptide encapsulation. According to the results, CH-HG-5 has the best cell adhesion ability although it's not 3D printable in Figure 4.38. However, there was no significant cell adhesion in CH-HG-3 and CH-HG-1 Figure 4.38 (C) and (D). Therefore, CH-HG-3 and CH-HG-1 were eliminated for the next steps and the KRSR peptide was encapsulated for HA-HG-5 and CH-HG-5. It's seen clearly that HA-HG-5 has the best result for hFOB 1.19 adhesion in Figure 4.39. That's why, CH-HG-5 was eliminated for the Alizarin Red S Staining mineralization test.

As seen in Figure 4.40, HA-HG-5 with and without KRSR peptide was compared to investigate the effect of KRSR peptide on mineralization on hFOB 1.19.

In conclusion, non-toxic, cell-adhesive biomaterial ink formulations were combined, characterized, and 3D printed. According to overall results, HA-HG-5 is chosen according to its 3D printability, toxicity, and cell-adhesive properties.

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