IZMIR KATIP CELEBI UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

NEUROVASCULAR Co-CULTURES IN HYDROGEL BUILDING BLOCKS

M.Sc. THESIS

Fulya ERSOY

Department of Biomedical Technologies

Thesis Advisor: Assist. Prof. Emel SOKULLU

APRIL 2018

IZMIR KATIP CELEBI UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

NEUROVASCULAR Co-CULTURES IN HYDROGEL BUILDING BLOCKS

M.Sc. THESIS

Fulya ERSOY

(Y140101004)

Department of Biomedical Technologies

Thesis Advisor: Assist. Prof. Emel SOKULLU

APRIL 2018

İZMİR KATİP ÇELEBİ ÜNİVERSİTESİ

FEN BİLİMLERİ ENSTİTÜSÜ

HİDROJEL BLOKLARDA NÖROVASKÜLER KO-KÜLTÜR ÇALIŞMALARI

YÜKSEK LİSANS TEZİ

Fulya ERSOY
(Y140101004)

Biyomedikal Teknolojileri Ana Bilim Dalı

Tez Danışmanı: Doktor Öğretim Üyesi Emel SOKULLU

NİSAN 2018

Fulya ERSOY an **M.Sc.** student of **IKCU Graduate School OF Natural And Applied Sciences** successfully defended the thesis entitled **"NEUROVASCULAR Co-CULTURES IN HYDROGEL BUILDING BLOCKS"**, which she prepared after fulfilling the requirements specified in the associated legislation, before the jury whose signatures are below.

Thesis Advisor:

Assist.Prof. Dr. Emel SOKULLU ……

İzmir Katip Çelebi University

Jury Members:

Prof. Dr. Bahattin TANYOLAÇ ……..

Ege University

Assist. Prof. Dr. Ozan KARAMAN ……

İzmir Katip Çelebi University

Date of Submission:

Date of Defense :

To my family,

FOREWORD

This thesis was supported by the University of Izmir Katip Çelebi, Department of Scientific Research Projects (BAP) and I would like to thank them for funding this research.

Foremost, I would like to thank my advisor Dr. Emel SOKULLU for her guidance, contribution, and support throughout the whole journey. I am deeply grateful her that being by my side and encouraging me in this way and leading an academic pathway.

I would like to thank Prof. Dr. Taner DAĞCI for his support during my research and providing all his resources when we are in need. I am also thankful to Prof. Dr. Gülgün ŞENGÜL for her support both financially and scientifically for letting me involve her project and widen my perspective in the field. My sincere thanks also go to Prof. Dr. Bahattin TANYOLAÇ who always share his valuable experiences with me and whenever I knocked his door always welcomed me and gave me advice when I am in need. I also wish to thank Dr. Ozan KARAMAN for his support during my research, particularly for cell culture studies.

I am particularly grateful my dear friends Ayşe KÖSE, Fazilet GÜRER, Öznur SİNGİN and Kübra ARSLAN who always supported me morally and scientifically for their contribution to my research.

Most importantly, none of this would have been possible without the love, patience and the support of my family who is always there to encourage me with their best wishes and advice. I owe a huge gratitude to my mother and father for allowing me and even cheering me up to dream high and work for it by heart to achieve my goals.

April 2018 Fulya ERSOY

TABLE OF CONTENTS

ABBREVIATIONS

3D: Three Dimensional

CAD: Computer-aided Design

DPBS: Dulbecco's Phosphate Buffer Saline

ECM: Extracellular matrix

FDA: Foof and Drug Administration

GelMA: Gelatin methacrylate

kDa: Kilodalton

NMR: Nuclear Magnetic Resonance

PBS: Phosphate Buffer Saline

PEG: Polyethylene glycol

PEGDA: Polyethylene glycol diacrylate

PGA: Polyglycolic acid

PI: Photo Initiator

PLA: Polylactic acid

PLGA: Polylactide glycolide

PU: Polyurethane

RGD: Arg-Gly-Asp peptide chain

TMSPMA: 3-(trimethoxysilyl)propyl methacrylate

USA: United States of America

UV: Ultra Violet

W: Watt

LIST OF TABLES

Page

LIST OF FIGURES

Page

NEUROVASCULAR Co-CULTURES IN HYDROGEL BUILDING BLOCKS

ABSTRACT

Creating a tissue or an organ model is the most emerging field of the biomedical research for the last decades. Three-dimensional scaffolds that will mimic the extracellular matrix were designed and used in various applications to investigate the behavior of cells.

In this study, both the synthesis of the polymer and the fabrication of three-dimensional hydrogel scaffolds were practiced. Gelatin methacrylate (GelMA) was synthesized prior to photopolymerization of the hydrogel. A novel scaffold fabrication device was designed and manufactured. This device allowed us fabricating computer aided designed geometries precisely by using cell encapsulated hydrogel solutions. Since co-culturing cells are challenging due to different growth rate and behavior in the culture medium, this device let us encapsulate different cells in different hydrogel posts while building a whole geometry.

The hydrogel texture characterization was performed using colored hydrogel solutions and it is observed that the device keep the distinct post in contact precisely. A neural tissue model was created using neural, epithelial and endothelial cells as a proof of concept. Cell distribution was evaluated using cell trackers that provide observing different cell types under fluorescent light and this proved that the fabrication system makes different cell type containing hydrogel posts one whole system. The viability of the co-cultured hydrogel system was investigated for seven days. Results showed that the viability of the system was above 80% which was a promising data for further studies. Overall findings showed that the precise hydrogel alignment device is promising for further application in many fields.

HİDROJEL BLOKLARDA NÖROVASKÜLER ALT-KÜLTÜR ÇALIŞMALARI

ÖZET

Doku veya organ modeli oluşturmak son yılların en gözde araştırma konularından biri olarak karşımıza çıkmaktadır. Bu amaçla, hücrenin ekstrasellüler matrisini taklit edebilecek üç boyutlu doku iskeleleri tasarlanmakta ve yapay çevreye farklı hücrelerin verdiği tepkileri incelenmektedir.

Bu çalışmada, hidrojel doku iskelelerinde kullanılan polimerin sentezi ve de bu hidrojel ile üç boyutlu doku iskelelerinin üretimi gerçekleştirilmiştir. Jelatin metakrilat (GelMA) polimeri sentezlenmiştir ve farklı desenlerde şekiller oluşturabilecek yeni bir mekanizmaya sahip olarak tasarlanan cihaz yardımıyla ve fotopolimerizasyon yöntemi kullanılarak hidrojel bloklar üretilmiştir. Bu cihaz, içine hücre hapsedilmiş hidrojel solüsyonları ile bilgisayar destekli tasarlanmış farklı desenleri oluşturabilmeyi mümkün kılmaktadır.

Cihazla üretilen hidrojel blokların karakterizasyonu için renklendirilmiş hidrojel solüsyonu kullanılmıştır ve her bir bloğun birbiriyle temas halinde olduğu gözlenmiştir. Sinir, endotelyal, epitelyal hücreler kullanılarak basit bir sinir doku modeli oluşturulmuştur. Hücrelerin jel bloklar içerisindeki dağılımı, flüoresan parçacıklarla izlenmiştir ve her bir hücre tipi ayrı bir hidrojel blok içinde olsa da bir bütün olarak bulundukları gözlenmiştir. Hücre canlılığı yedi günlük bir test ile değerlendirilmiştir. Hücre canlılığı %80'in üstünde gözlenmiştir. Farklı hücrelerin sahip olduğu farklı büyüme hızı ve kültür ortamındaki farklı davranışlarından dolayı hücrelerin alt-kültürü oldukça zor bir süreçtir ancak bu cihaz farklı hücre tiplerini ayrı hidrojel bloklarının içine hapsetmeyi sağlayıp aynı zamanda da bütün bir şekil oluşturmayı mümkün kıldığından alt-kültür sistemini kolaylaştırmaktadır.

Tüm analizler dikkate alındığında hidrojel hizalama cihazının çeşitli çalışma sahalarında kullanılmak üzere oldukça gelecek vadeden bir tasarım olduğu görülmüştür.

1. INTRODUCTION

The human body is a system that has three-dimensional structures and consists of various types of cells. The skeleton system and the connective tissues let the body endure its three-dimension structure and all organs function as three dimension systems. So, when modeling a tissue or an organ in vitro was considered, the importance of the three-dimensional platforms must be well-understood. That is why tissue engineering approaches focus on creating supports for cells and making them behave as they do in the body.

Creating a treatment or a replacement for injured body parts is the main goal of the tissue engineering. Recently, tissue engineering is the most emerging field and gains a lot of attention from scientific community, governments and media. It is believed that a fully functioning organ can be engineered via harvesting a patient's own cells, culturing them in vitro and transferring them into a porous three-dimensional scaffold. However, there are obstacles since the biological environment of the organs and the way of their functioning mechanism was not thoroughly understood. The main bottleneck of the tissue engineering is the fabrication of the "*best*" scaffold which provides the biocompatibility (creating no host reaction when in contact with the body), biodegradation (being able to degrade in time while replacing the tissues own extracellular matrix and not producing any toxic degradation residues), porosity and permeability (required for the vascularization, nutrient and waste transfer), mechanical strength and surface environment that supports cell attachment and proliferation [8]. The requirements for an appropriate scaffold are complex and yet not fully discovered.

The main issue for designing a proper scaffold is the material properties. In this case, choosing a suitable material is of great importance. Presumptive materials are ceramics, synthetic and natural polymers, metals and the mixture of these materials [53].

Metals and ceramics made a great contribution to medicine as orthopedic replacements. Stainless steel, cobalt-based and titanium-based alloys [51] were the metals that are used for implantation procedures. On the other hand, the ceramics that were typically used were bioglass, zirconium, aluminum and calcium phosphate [24]. Hip joints were the most

abundantly applied prosthesis that improves many people's life quality [53]. However, except the bio ceramics like tri-calcium phosphate derivatives they are not biodegradable and due to material properties, due to it is hard to shape and process them. Considering these major handicaps, the scientists directed their attention to the polymeric materials and their promising characteristics.

Polymeric materials were being broadly used for tissue engineering applications. Natural polymers such as gelatin, collagen, chitin, chitosan, etc. and synthetic polymers like polylactic acid (PLA), polyethylene glycol (PEG), polyurethane (PU) were used widely as scaffolds to repair the skin, cartilage and bone injuries. Both natural and synthetic polymers have pros and cons. Natural polymers provide a great replacement for the extracellular matrix since their origin is from nature and they have the components that already exist in the body. However, they are lacking the mechanical strength to be applied to bone or vascular tissues, which are subjected to great mechanical stress. By the way, the chemical composition of the polymer may differ from batch to batch and so the reproducibility is an issue for natural polymers, as well. On the other hand, synthetic polymers are easy to control and process a, their mechanical strength can be tailored. They also have a significant benefit of availability and reliability, but due to their chemical structure they are not easy to degrade and might have toxic degradation products[25].

1.1. Three-Dimensional Tissue Engineering

The fundamental goal of the tissue engineering is mimicking the extracellular matrix both physically and chemically. Organizing cells into a three-dimensional construct and stimulate them to grow into a functional tissue requires a well-designed scaffold. Scaffold architecture is the actual issue to manufacture an appropriate tissue construct. Since the main goal of tissue engineering is mimicking *in vivo* environment and achieving functional tissue models *in vitro,* providing a three-dimensional support, which makes cells act as they are in their own extracellular matrix is essential.

Cells are in contact with the extracellular environment in the body and they have both mechanical support and nutrient transfer from this environment. Scientists were creating

three-dimensional cell culturing methods to provide this 3D surrounding *in vitro*. There are three approaches so far. Acellular scaffolds were fabricated with various techniques such as salt leaching [23; 39], gas foaming [68], phase separation [42], freeze drying [23], 3D printing [61], stereolithography [36], electrospinning [69] etc. and cells were seeded on to them to create three-dimensional tissue models. In another approach, hydrogel-cell hybrid constructs were fabricated via cell encapsulation method [49]. Cells are mixed in the pre-polymer solution and crosslinked while cells are already inside the solution. Besides these approaches, scaffold-free cell aggregations were also formed [12] to create 3D tissue constructs.

1.2. Parameters for Designing a Scaffold

Scaffold fabrication is a challenging process due to the complexity of the human body. There are plenty of parameters must be considered to achieve the tissue integrity and regeneration. Since most of the parameters are related to material, selecting a suitable material is the most important part of the scaffold fabrication. The main parameters (Figure 1.1) are;

- Biocompatibility
- Biodegradability
- Porosity
- Permeability
- Mechanical strength and flexibility
- Surface adhesion of the cell attachment

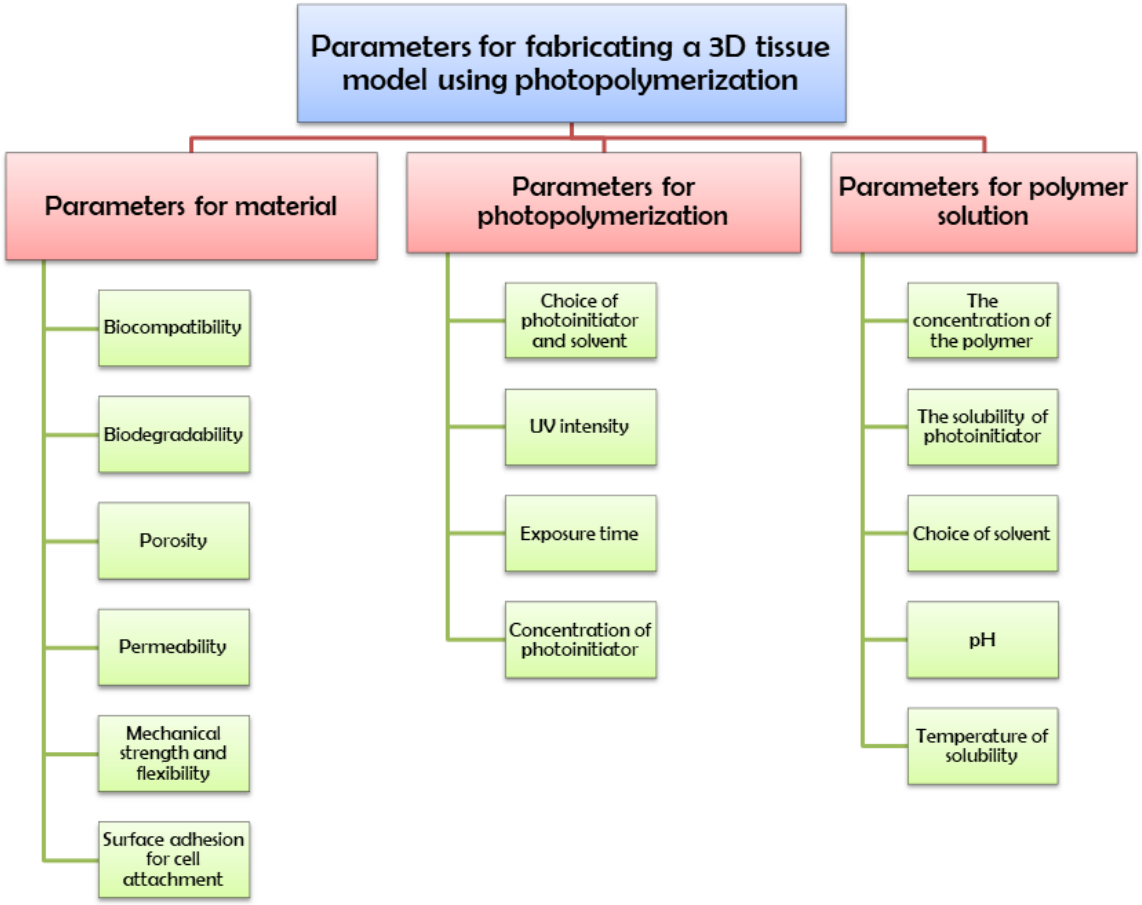

Figure 1. 1: The schematic of the modeling parameters

Biocompatibility means that the material should not be toxic and will not cause any immune response when it is interacting with the human body [53]. This is the most selective parameter prior to fabrication. Biodegradation is the act of disintegration of the material in body environment while replacing with the extracellular matrix. As cells are secreted their own extracellular matrix, it is expected that the scaffold gets degraded and while the material degrading, its required that it remains its biocompatibility and produces nontoxic residues [21, 53].

Porosity is a constitutive feature of scaffold architecture. This is essential for cell migration, nutrient transfer, and waste removal. Pore size and the distance between pores steers the

vascularization and cell migration in the scaffold. At least 90 % porosity and 100-400 nm pore size are the required numbers for the desired regeneration in tissue scaffolds [4].

The permeability of the material supports the diffusion of the nutrients into scaffold and wastes out of the scaffolds. Permeability provides the nutrient exchange while the extracellular matrix is being built around the cells and allows signaling molecules to circulate in the tissue construct. The more porosity means the more permeability within the scaffold [8].

1.3. Hydrogels

Hydrogels are the type of polymers that have the ability of gelation. They are an appropriate source of soft tissue scaffolds due to their structural similarity to the extracellular matrix, biocompatibility, and hydrophilicity which results in the high water content (≥30 % by weight) [30, 38].

Both natural and synthetic hydrogels were applied in medical history. Due to their controllability and reliability, synthetic polymers were broadly applied by the surgeons. Some aliphatic polyesters such as polylactic acid (PLA), polyglycolic acid (PGA) and the copolymer of these (PLGA) were approved by FDA for many applications and have been widely used as contact lenses, skin grafts etc. [62,66]. On the other hand, natural hydrogels like collagen, alginate, gelatin, hyaluronic acid, agarose etc. are promising sources for scaffold design. Some of these hydrogels such as collagen and hyaluronic acid are already existing macromolecules in the extracellular matrix. Hence, they provide desired biological properties for the cell adhesion, proliferation, migration, differentiation, and vascularization. Their biodegradability is another desired parameter and can be via altering the degree of crosslinking [38, 39].

Gelation process can be via temperature alteration[5] , pH changes[2] or photo polymerization[47]. Temperature change was an issue due to protein deterioration at high temperatures, however, studies on decreasing the temperature difference as close as body temperature brought successful results[11]. pH change is another approach for gel

crosslinking where alginate hydrogels were formed via this method. Even though the method was easy to perform it is not providing control over the crosslinking density and gel forms in a toxic environment[2]. Photopolymerization is another technique where polymers were reacted with acrylates or methacrylates prior to light exposure. Then, in the presence of a photoinitiator substances, photocrosslinking occurs[47]. Once the crosslinking completed, the photoinitiator must be washed off from the environment in order to remove toxic chemicals. Photopolymerization is one of the most preferred methods to create gels due to it controllable parameters and relatively less toxicity. In addition to all these parameters the scaling- up the fabrication of the scaffold, being able to manufacture the scaffold in desired geometries and sterilize it are essential features of the scaffold design.

1.4. Photopolymerization

Light sensitive substances called photoinitiators (like Irgacure 2959) interact with visible or UV light and then they create free radicals that initiate the crosslinking of the liquid pre-polymer solutions into gel form[59]. Polymerization or curing the materials using light has been used broadly in dentistry to seal the dental prosthesis and dental cavity fillers *in situ*[18, 45]. Photopolymerization has an extensive application area such as membranes, coatings, printing materials, surface modifications and polymeric materials[47]. Including fast curing time (from a few seconds to a minute), temporal and spatial control over curing, appropriate temperature requirements (room temperature or physiological temperature conditions) and negligible heat production, photopolymerization has a great advantage over the conventional methods. Besides, photopolymerization provides users an invasive approach during the application. The pre-polymer liquids can be injected to the location where it is needed using catheters[65], laparoscopic devices[27] or just with a syringe[14] to the dermal tissue to perform an *in situ* polymerization. This facilitative approach makes the technique attractive for biomedical application as it allows the practitioner to create complex architectures that adhere and adapt to the tissue construct. However, since the biological environments require a limited range of temperature and pH levels, as well as no presence of the toxic materials (such as organic solvents or monomers), still some photopolymerization systems fulfill the

requirement of the biological systems by means of relatively mild conditions (lower light intensity, body temperature, shorter exposure time) that allows to process to be carried out with cells.

1.4.1. Photopolymerization Technique

This method can be explained as light-guided polymerization and it is proven that it is a promising tool for cell related studies especially examining three-dimensional cell behavior, as well as for many other biomedical applications[9, 14, 18, 44, 46]. Combination of the irradiation with suitable light wavelength and the presence of distinct substances that absorbs light initiate the radical photopolymerization. The light absorbing substances then either decomposes or eases the decomposition of co-initiator substances, into free radicals that drive the polymerization. Depending on their radical formation mechanism following photon absorption, the radical photopolymerization systems are branch into two main classes. First class (cleavage type) photoinitiators decompose into two radicals after the absorbing the photons. On the other hand, second class photoinitiators stay in an excited state following photon absorption, remove a hydrogen atom from a co-initiator substances[18].

Camphorquinone-amine combinations[6] and eosin Y[10, 56] were applied as second type photoinitiators and showed some successful cell encapsulation applications[45-47]. Even though these photoinitiators have high water solubility, they have practical drawbacks including the requirement of a second co-initiator, overlapping excitation and emission spectra of eosin Y with many fluorophores that were widely used for cellular imaging. Most of the first class photoinitiators, that have visible light absorbance, have poor water solubility and high cell toxicity[10]. The most commonly employed UV photoinitiator for cell encapsulation is 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propanone (AKA Irgacure 2959) despite its poor water solubility. Even though it has the highest water solubility among the other first-class photoinitiators, its water solubility at optimal conditions is less than 2 % (w/v)[19]. On the other hand, even preparing a 0.5% soliton requires a considerable agitation and heating to dissolve the Irgacure thoroughly. To achieve an efficient polymerization, the absorbance spectrum of the photoinitiator must be demonstrated

an acceptable overlap with the emission spectrum of the selected light source. However, for cell related studies, low wavelength light sources must be excluded due to their phototoxic and mutagenic features[31, 33]. Mostly the light emission is filtered to make light centered around 365 nm, but the molar extinction coefficient of Irgacure at 365 nm is pretty low ($4M^{-1}cm^{-1}$) and tails off almost entirely before reaching to 370 nm[16]. Hence, this limits the use of Irgacure 2959 with a different wavelength, however; it still is a great option for UV photopolymerization for cell encapsulation.

1.4.2. Photopolymerization in Tissue Engineering

Photopolymerization of monomers was investigated widely for both UV and visible light. Even though these systems work quite well for plenty of applications, they are not appropriate for tissue-related practices due to monomeric cytotoxicity. Thus, generally, macromolecular precursors were used as photopolymerizable hydrogels for tissue engineering applications. The examples of these macromolecular polymers are polyethylene glycol acrylate or methacrylate derivatives, hyaluronic acid derivatives, dextran methacrylate derivatives that are water-soluble molecules, which have two or more reactive parts.

There are three main practices of the photopolymerizable hydrogels in biomedical applications. These are using hydrogels as barriers after the injuries to enhance the healing process, drug delivery carriers and cell encapsulation precursors. The hydrogel barriers were constructed from the biodegradable poly (ethylene-glycol-co-lactic acid) diacrylates as a coating macromers on the injured tissue surfaces. This approach was aimed to prevent thrombosis and restenosis[27, 34]. In another application photopolymerization of this macromolecular hydrogel was used to prevent post-operative adhesion formation[57].

Hydrophilic macromolecular drugs like proteins and oligonucleotides can be carried with hydrogels. Hydrogels are a great source for drug carrier due to their biocompatibility, ease of controlling the crosslink density and swelling and degradation rate via altering the fabrication parameters. On the other hand, since the photopolymerization can be performed in situ, the photopolymerizable hydrogels are attractive for local drug delivery applications[1,7]. They are also used for intravascular drug delivery using interfacial photopolymerization[1].

Encapsulating cells to provide an immunoisolation while maintaining the diffusion of oxygen and nutrients is another usage of photopolymerized hydrogels. The cured polymers allow selective cell transfer to the desired target. Polyethylene glycol diacrylates (PEGDA) were used as photopolymerizable hydrogels to develop an artificial pancreas tissue[9, 10, 50]. PEGDA pre-polymer solution that contains islets were used to create microspheres that provide immunoisolation and the diffusion was optimized via decreasing the thickness of microspheres. As a result, the high viability of islets was observed after 30 days of transplantations in rats[9].

Hydrogels are a great source for soft tissue engineering due to their mechanical and structural properties. Hence, photopolymerizable hydrogels are attractive as tissue scaffolds. They were studied for cartilage tissue regeneration[15], vascular smooth muscle cells[45], vascular tissue with endothelial cells[60] and neuron development[22]. Photopolymerizable hydrogels also allow encapsulation of bioactive molecules alongside the cells to manipulate the cell regeneration or differentiation[45].

1.4.3. Photopolymerization Parameters for Tissue Engineering

Photopolymerization is a crosslinking technique that is used either to polymerization of monomers or macromolecular polymers. In any case, a light reactive photoinitiator molecule is required to initiate the process. The better crosslinking density depends on proportional to the better photoinitiator concentration and the radiation density. For photopolymerization, high-energy radiation[54, 55], UV radiation[43] and visible light radiation[41] was investigated and so far, better results were reported for UV radiation considering cell viability and cytotoxicity.

Long-wave UV photopolymerization is one of the most common approaches to hydrogels for biomedical applications due to advantages of spatial and temporal control of the process, low energy requirements and medically acceptable curing times. UV photo-crosslinking can be carried out at body temperature and pH, both in vivo and in vitro. Since the photopolymerization is driven by free-radicals from photoinitiator molecules, the non-toxicity of these molecules is another requirement[46]. The concentration and homogeneity

of radicals provide an opportunity for tuning the porosity and crosslinking density. However, the presence of the free-radicals brings the possibility of cell interaction and this may cause cell damage during the reaction. On the other hand, the choice of toxic solvent and photoinitiator, high intensity of UV light, heat formation during the polymerization may potentially harm the cells[6, 47]. The concentration of the polymer and photoinitiator solution has a great role in both cell survival and tuning the crosslinking density, as well.

As it is shown in figure 1 the main photopolymerization parameters for biomedical applications are a selection of polymer, photoinitiator and solvent, optimization of UV intensity and exposure time and the concentration of polymer and photoinitiator solution.

1.4.4. Spot Light UV Curing Systems

Photopolymerization can be performed either with visible light or UV light. In this study, UV photopolymerization of hydrogels was performed. The UV curing system has a 200 W UV curing lamp and provides a spot focused UV light that is advantageous for a photomask using applications. Spot size can be altered by changing the distance from probe to the subject. It is possible to use collimating lenses to enhance the spreading of light to the subject. The UV intensity can reach up to 30W/cm^2 and can be applied as low as 2 mW/cm^2.

1.4.5. Polymer Solution Parameters

Gel fabrication with photopolymerization method requires a pre-polymer solution which contains a photoinitiator. There are a couple major parameters that determine gel quality and suitability for cell experiments. These are photoinitiator solubility, polymer concentration, solvent and pH of the solution.

Photoinitiator Solubility The best photoinitiator for UV photopolymerization is Irgacure 2959 considering cell viability. However, Irgacure has very low water solubility at physiological conditions and even under high temperature and vigorous mixture it is hard to dissolve it in water[19]. So, while preparing the polymer solution, the first step is making sure that the photoinitiator has been dissolved completely.

Polymer Concentration The concentration of the polymer solution affects the rigidity, stiffness, and porosity of the gel. While determining the polymer concentration, the cell migration and proliferation throughout the gel must be considered. If the gel is too stiff, cells cannot move in the gel and this is not the desired situation for tissue and organ modeling. Besides, polymer concentration has a role to decide the UV exposure time. The more concentrated the solution, the less the UV exposure needed.

Solvent On the other hand, choosing the solvent to prepare polymer solution is crucial for cell viability. Most of the organic solvents are highly cytotoxic; therefore, the polymer solution must be prepared with non-toxic solutions such as water or phosphate buffer solution.

pH Cell experiments are performed under physiological conditions to mimic body environment in vitro. One of these conditions is the pH level of the solutions. In the body, the main carrier is blood and all the nutrients, waste, oxygen and carbon dioxide were carried via blood. The pH level is the blood is 7.4 and this is the level that all the body environment mimicking solutions must have. When the polymer dissolution occurs in the solvent, the pH level must be maintained around physiological levels to maintain cell viability.

1.5. Gelatin

In tissue engineering, mimicking extracellular matrix is the main aim to create an ideal scaffold. Extracellular matrix is a composition of extracellular molecules that are secreted by the surrounding supportive cells. This matrix consists of two major

macromolecules which are proteoglycans such as glycosaminoglycans, perlecans, aggrecans etc. and fibrous proteins such as collagen, fibronectin, elastin, and laminin (Figure 1.2) [20].

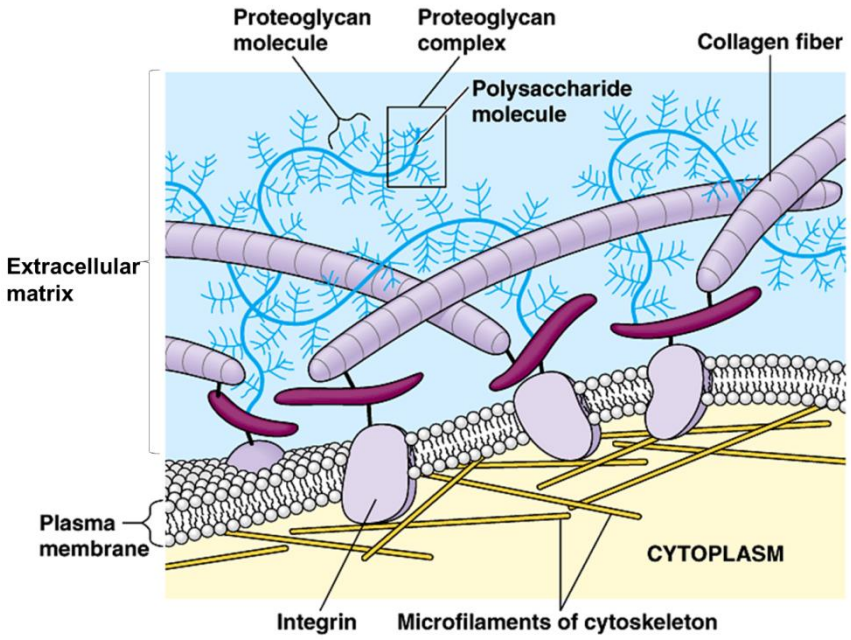

Figure 1. 2: Extracellular matrix[52]

Collagen is the most abundant fibrous protein in an extracellular matrix structure. It is also the major protein constituent of connective tissue, bone, skin, and cartilage. Even though this makes collagen a great scaffold source, collagen has antigenicity because of its biological origin and there is a huge risk of triggering host reaction[3].

Gelatin is a derivative of collagen that is obtained via hydrolysis and its composition is almost identical to collagen. So, this makes gelatin a great alternative for tissue engineering applications. Collagen has a triple helix structure, however, during thermal denaturation or degradation (physical or chemical) of collagen, this triple helix structure gets broken and this makes gelatin a biodegradable and nonimmunogenic product[26]. Gelatin has relatively less antigenicity compared to collagen though, the signaling molecules such as Arg-Gly-Asp (RGD) sequence of the collagen that stimulate and promote cell attachment, differentiation and proliferation still exist in the composition of gelatin[40].

Gelatin is soluble in aqueous solutions and in liquid form at physiological temperature. Hence, for long-term experiments, gelatin must get crosslinked prior to experiment so, both

the mechanical and thermal stability will be enhanced and cell experiments will be performed in a more stable environment. Gelatin can be crosslinked via using chemical crosslinking agents[3, 32, 40] or photocrosslinking can be performed after methacrylation of the gelatin[48].

1.5.1. Gelatin methacrylate (GelMA)

Photocrosslinking requires methacrylate or acrylate molecules which bonds open with the presence of photoinitiator molecules and create new bonds under the light exposure. As a brief definition, gelMA is a gelatin derivative, which consists largely of methacrylamide groups and a relatively small amount of methacrylate groups. GelMA must be submitted to photopolymerization to build a covalently crosslinked gel product.

GelMA was first fabricated by Van Den Bulcke et al. and despite the minor variations of the protocol, its principle is same[64]. Briefly, the protocol is the direct reaction of gelatin with a methacrylic anhydride in phosphate buffer saline (PBS) at 50°C. In this reaction, the amine and hydroxyl groups of amino acid residues were substituted with methacryloyl groups (Figure 1.3). Mechanical properties of the gelMA can be tuned by changing the amount of methacrylic anhydride that added to the reaction. On the other side, the substitution degree of amine and hydroxyl groups can be enhanced via maintaining the pH level high[28]

.

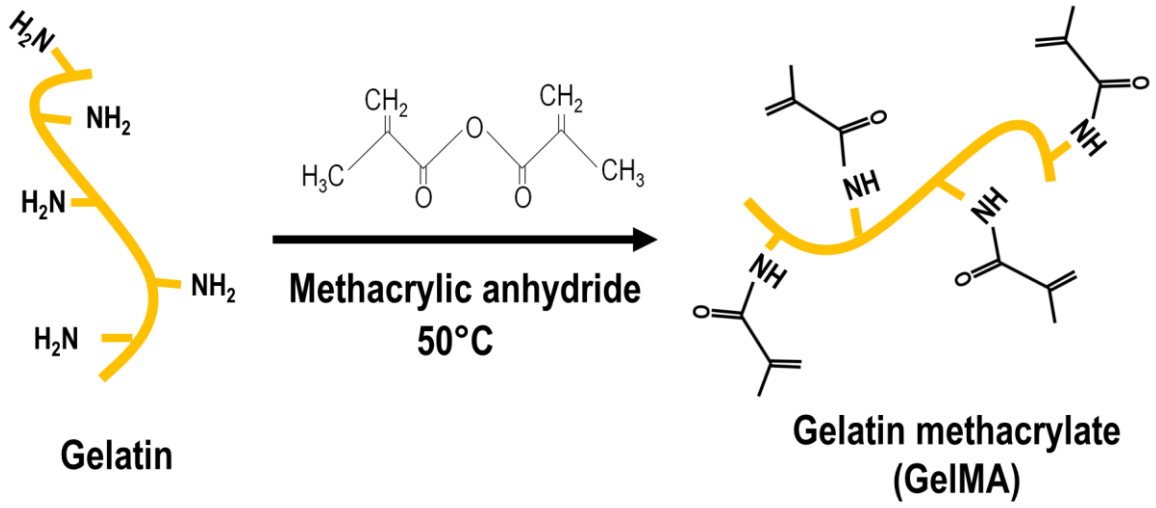

Figure 1. 3: The synthesis of gelatin methacrylate[67]

The substitution reaction typically stopped by diluting five times with PBS and then, the solution was subjected to the dialysis process in dialysis tubing via passing deionized water through for at least a week to remove the cytotoxic component completely. The final solution was freeze-dried and stored. Since the reaction of methacrylic anhydride and gelatin is a two-phase reaction where methacrylic anhydride added and dispersed into liquid solution, the way methacrylic anhydride added into the solution and the mixing may affect the quality of final product and the degree of reaction[70].

1.5.2. Three-dimensional GelMA gel construct fabrication

The organs and tissues in the body have a three-dimensional (3D) environment around them and they function as three-dimensional systems. Hence, while trying to model a tissue or an organ, this three-dimensional system must be considered and modeled accordingly. Fabricating three-dimensional scaffolds serves as the 3D environment of the cells inside the body. In this research, gelatin methacrylate was chosen as a material to fabricate the 3D supportive construct. For manufacturing 3D gels photocrosslinking method was used and UV photocrosslinking was performed. The advantage of gelMA three-dimensional constructs can be told as;

- Physical and mechanical support for cells
- Mimicking the 3D environment of the ECM
- Promoting cell adhesion and differentiation via RGD proteins that already exist in GelMA
- Providing a porous environment for cell migration and proliferation.

3D gelMA constructs can be fabricated by exposing the UV light directly to the pre-polymer solution or computer-aided designed photo-opaque masks can be used to create different textures. Using photomasks allow us to create geometric shapes and build various types of models. For example, a square can be divided into 4 identical squares and 4 different parameters can be worked at once with one complete set of texture (Figure 1.4). On the other hand, photomasks allow working with different cell types at once in one complete geometry, as well[22].

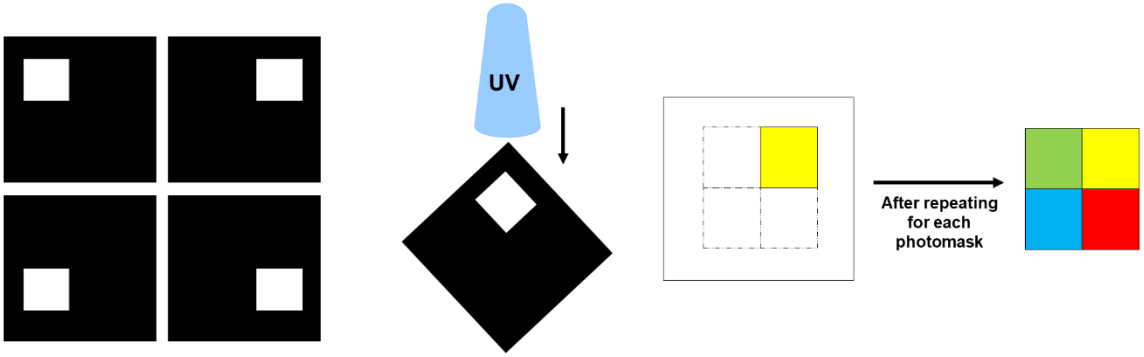

Figure 1. 4: Creating a full geometry with distinct gel blocks

1.6. The Importance of Co-Culturing Cells

Co-culturing different types of cells were always attractive for modeling a complete tissue and organ. In the body, the cell viability, differentiation, migration, proliferation, apoptosis, and even phenotypical features are all controlled via signaling molecules. The most studied signaling molecules are integrins and cytokines. Integrins are the most abundant transmembrane proteins and they regulate both the cellular binding to ECM and response to surrounding ECM signal proteins coming from different cell types. There is a phenomenon

that cells are crosstalking to each other using signaling proteins and decide how they will act in their environment. Therefore, not only mimicking the ECM is essential for an appropriate modeling but also co-culturing various types of cells are crucial to achieving a functional in vitro tissue and organ modeling.

When cell lines are cultured in vitro, they may lose their phenotypic features or even cellular characteristics. Since signaling molecules control the maintenance of cellular features, lacking crosstalk may be causing these issues. As various cell types exist together in vivo, distinct signaling molecules from each type of cell can possibly control the behavior of each cell. In monoculturing, the functioning of cells is limited due to lacking signaling molecules. On the other hand, since the information about the crosstalking mechanism and the signaling molecules are limited, we are not able to mimic the in vivo environment completely in monoculturing. However, it is possible to improve in vitro culturing conditions via co-culturing. While three-dimensional scaffolds are mimicking ECM, co-culturing different cells may provide the lacking crosstalk and allow constructing a better in vitro model.

1.6.1. Co-culturing Parameters

Co-culturing systems are challenging mechanisms and there are a few parameters must be considered. They are:

- Choosing cells
- Choosing culture medium
- Cell type characterization
- Seeding strategy
- Dynamic or static culture
- Distribution or alignment in a culture dish

Before seeding cells into a culture dish, cell source must be decided whether a cell line or primary cells will be used. Once the cells were chosen an optimal cell culture medium which will satisfy all cell types must be optimized prior to culturing. Depending on the tissue or organ that will be studied, type of cells must be determined. Some cells proliferate faster than others while some of them are more fastidious than others. Hence, while deciding the seeding

density, the ones that are proliferating faster and more tolerant are better to be at lower concentrations. All cells are subjected to a different level of mechanical stimulation such as compression, shear or tension forces in vivo, so cells must be submitted to proper mechanical stress in either static or dynamic conditions. This mechanostimulation
can be a light agitation during carriage or a dynamic compression stress in a bioreactor depending on the tissue type[29, 35].

2. MATERIALS AND METHODS

2.1. Devices

- Omnicure S2000 Spot light UV curing device (Excelitas Technologies Corp., USA)
- A light microscope (Olympus CK2, USA)
- Centrifuge (Thermo fisher, SL 16R, USA)
- CO2 incubator (Thermo Fisher, FORMA Series II Water Jacket, USA)
- Class II laminar flow hood (NUAIRE Class II, MN, USA)
- Water bath circulator (Commat, WBC3044, Turkey)
- Inverted fluorescent microscope (Olympus CKX53, USA)
- 1-10, 20-200 and 100-1000 µl micropipettes (Thermo fisher, USA)
- Hemocytometer (Neubauer)
- 22 x 22 mm no:1 coverslips (Isolab, Canada)

2.2. Methods

5 main steps were followed in this study to complete the three-dimensional neural co-culture experiment.

1. Manufacturing 3D gel fabrication device
2. GelMA Synthesis and characterization
3. The hydrogel characterization of digitally created geometries
4. Cell viability tests for co-cultured hydrogel posts
5. Functional immunostaining of hydrogel posts

2.2.1. Manufacturing the 3D gel fabrication device

A novel device that allows fabricating precise hydrogel blocks using photomasks was designed. This device has three main parts, which are the main case, mask holder and mask frame (Figure 2.1). This whole system was designed as a drawer-like system (Figure 2.2.5.1-1) and limiting the mobile parts of the system made it more precise for the alignments while creating co-cultures where the alignment of the distinct parts of the geometry is critical for

the communication between cells. The device was designed using a CAD software (SolidWorks 9.9.14, USA) and was manufactured in Yönsis Lazer, İzmir, Turkey. 304 stainless steel was used as a material and the device was assembled using a cyanoacrylate adhesive (Cartell 911, Taiwan). Photo-opaque masks were designed using graphical design software (CorelDRAW, Corel corp., Canada) and printed out by A print İzmir, Turkey using a chrome based printing technique.

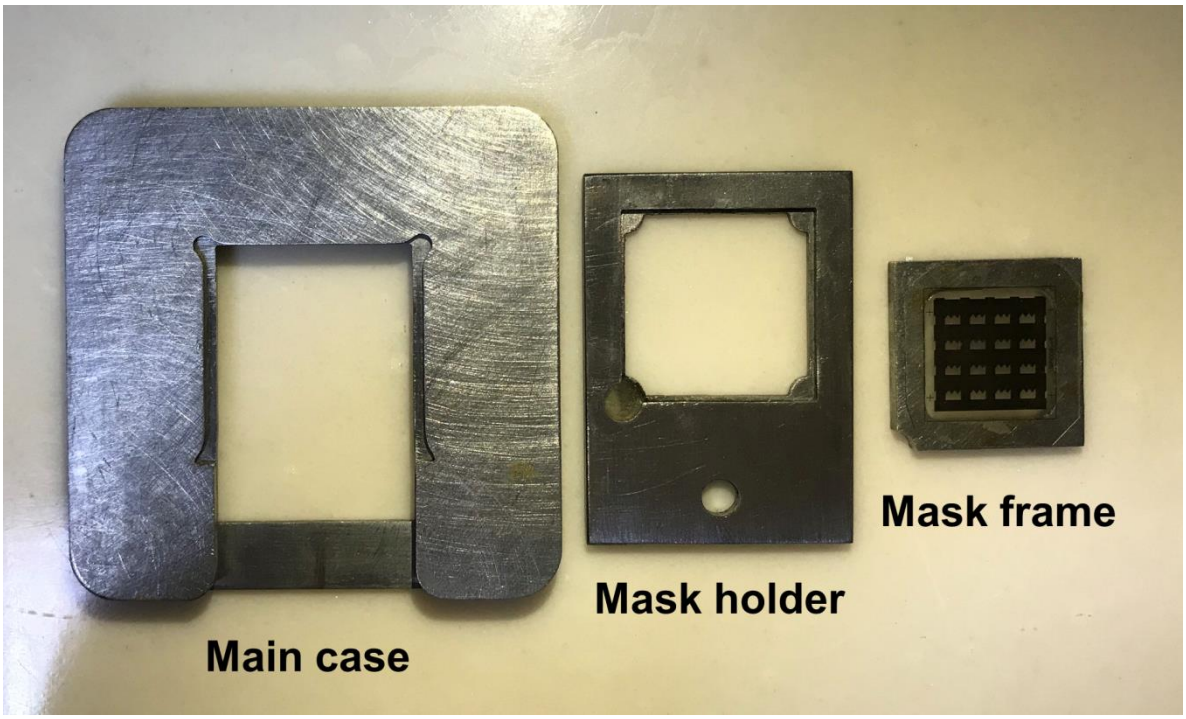

Figure 2. 1: The elements of the 3D gel fabrication device

2.2.2. Gelatin methacrylate (GelMA) Synthesis

Gelatin (Fluka Analytical, Germany) was added into 100 ml of Dulbecco's phosphate buffer saline (DPBS) (Bioshop, Canada) at a concentration of 10 % (w/v) and dissolved using magnetic stirrer at 60°C. When the solution became clear, methacrylic anhydride (Sigma Aldrich, Germany) at the concentration of 8 % (v/v) was added with a rate of 1 ml/min. The solution was kept at 50°C for 3 hours under a dynamic stirring for the reaction. The reaction was stopped via adding 5-fold dilution of the solution with pre-warmed (40°C) DPBS. The

solution was dialyzed for 1 week in distilled water with 10 kDa cut-off to remove salts and unreacted compounds. The polymer solution was then lyophilized for a week to obtain porous foam and it was stored at -80°C.

2.2.3. Surface treatment of coverslips

Coverslips were acrylated to enhance the adhesive properties of the glass. This acrylation process was performed using 3-(trimethoxysilyl)propyl methacrylate (TMSPMA) (Sigma-Aldrich, China). Briefly, coverslips were wetted with 10% sodium hydroxide (Merck, Germany) solution for 1 hour, washed with distilled water and dried using nitrogen gas. The coverslips then dipped into TMSPMA overnight at 80°C. The treated coverslips were washed with ethanol 3 times and dried using nitrogen gas. The coverslips were kept at room temperature prior to experiments. All the treated coverslips were used within 7 days after acrylation.

2.2.4. Preparation of pre-polymer solution

Pre-polymer solution was prepared fresh prior to UV curing process. Photoinitiator (PI) 2-hydroxy-1-[4-(hydroxyethoxy)-phenyl]-2-methyl-lpropanone (Irgacure 2959) was purchased from Boysan Boya Sanayi Ticaret A.Ş., Istanbul. PI was dissolved in DPBS (Bioshop, Canada) with the concentration of 1 % (w/v) at 80 °C. 5% (w/v) GelMA was added to solution and then vortexed. Since the solution was light sensitive, it was kept in dark during the study.

2.2.5. 3D gel fabrication system

The fabrication system consists of five main elements (Figure 2.2). These are:

1. 3D gel fabrication device
2. UV probe holder
3. UV probe
4. Device carrier
5. OmniCure UV light spot curing source

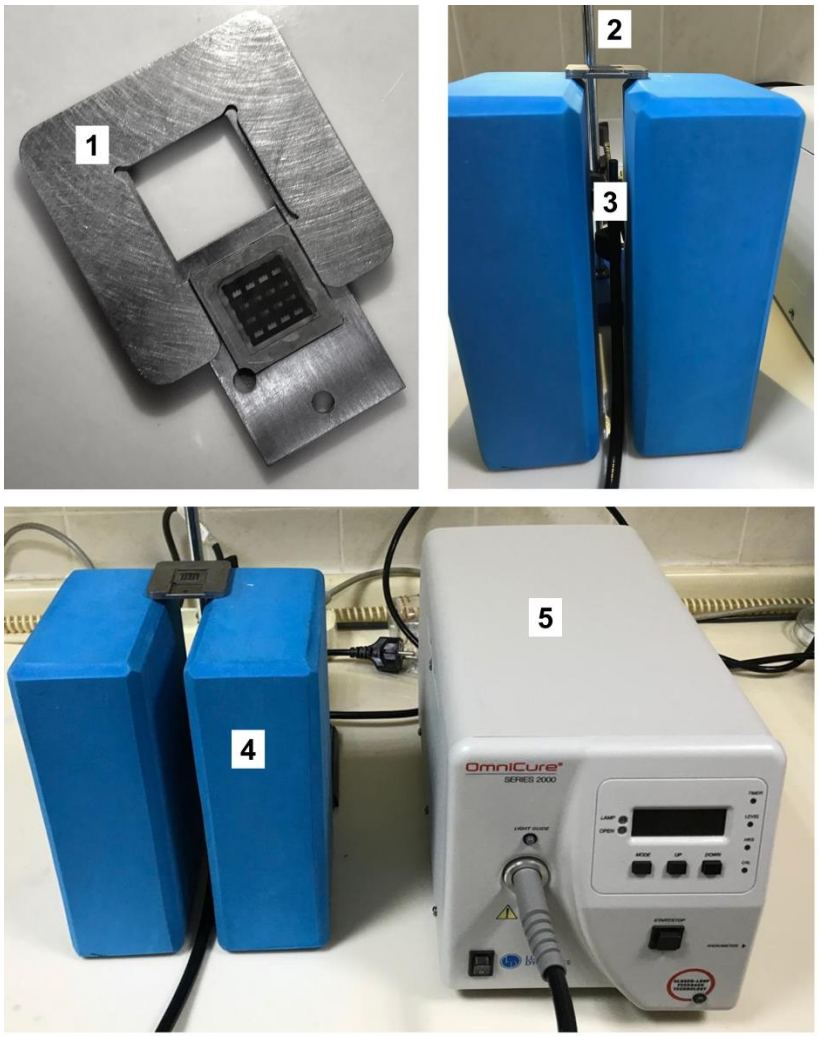

Figure 2. 2: The 3D gel fabrication system

2.2.6. Fabrication of hydrogel posts

Six different geometries were designed digitally based on squares, circles, and texts. These geometries were sectioned into elements (concentric circles, saw-like fractioned square, complementary checkerboard-like square, microscale text (IKC) and radially sectioned circles) to create colored images and co-cultures. Photomask sets were designed for each element to create individual geometries. The number of photomasks differed for each set depending on the elements that each geometry had. Photomasks were designed so that when they were placed on top of each other as they will make connected and concentric patterns.

Mask alignments were achieved using the crosses that are located at each corner of the photomasks which are drawn with 9 mm separation and the black frame that aligns with the inner square of the photomask frame was designed as 12 mm by 12 mm.

An alignment device was designed to achieve precise digitally formed patterns. Since the precision is the most important parameter to create definite geometries, minimum mobility was aimed at the design. A drawer-like system was created to make the process convenient and practical. The device was designed based on regular 22 mm by 22 mm cover glass and the elements of the device assembled using cyanoacrylate adhesive glue (Cartell 911, Taiwan). The only mobile unit of the device was mask holder to enhance the precision of the process. The photomasks that were created for each unit of the patterns were stabilized on the mask frames using low- viscosity adhesive (Cartell 911, Taiwan). To maintain the precision photomask frame was placed on to mask holder and mask holder was pushed into the main device like a drawer (2.2.5.1-1). TMSPMA treated coverslip was then positioned on to device prior to UV treatment. Since the device was molded precisely to be able to fit the 22 mm by 22 mm coverslip, the coverslip was perfectly stabilized on the device. A spacer involved coverslip was designed and cut from polystyrene plate which is a hydrophobic material that will not let hydrogel posts cure on it. Once the coverslip and mask holder was stabilized the fabrication procedure was followed (Figure 2.3).

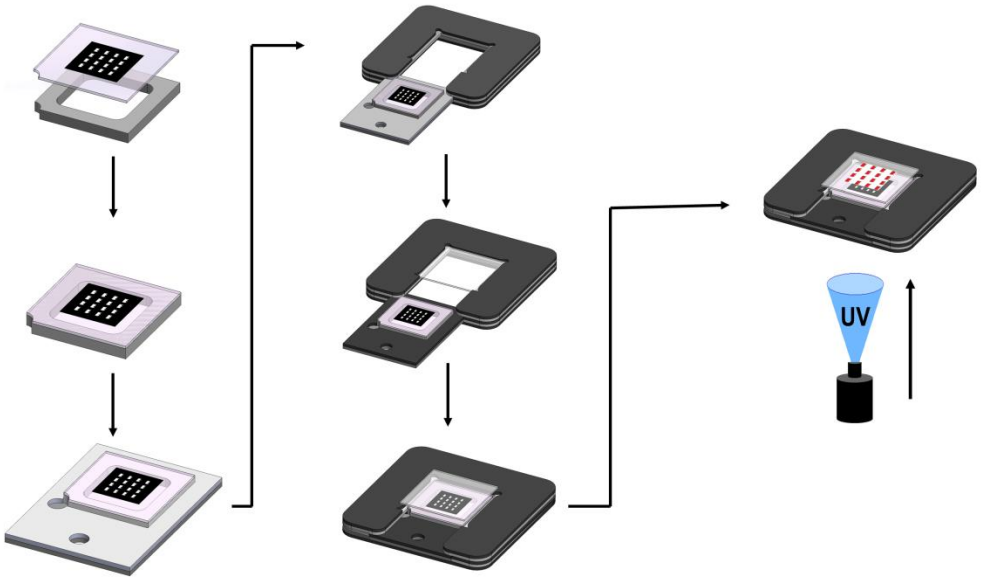

Figure 2. 3: The working process of the 3D gel fabrication device

2.2.7. The fabrication procedure of 3D gelMA posts

The following steps were followed to fabricate a complete geometry:

1. To fabricate the first unit of certain geometry 35 µl of the pre-polymer solution was pipetted on to TMSPMA coated coverslip and then covered with spacer cover.
2. The device was exposed the UV light using The OmniCure S2000 UV Light Curing System (Excelitas Technologies Corp., Waltham, MA, USA). The device was located at a distance of 60 mm from UV probe and power density was set to 6,25 W/cm^2. UV exposure duration was set to 35 seconds for cell encapsulating hydrogel posts, but for the colored hydrogel posts, the duration was varied (Table 2.1).
3. After UV exposure, the uncross-linked pre-polymer solution was removed via washing with DPBS.

4. To fabricate the second unit of the final geometry, mask holder was pulled out and mask frame was replaced with the second photomask, then mask holder shoved into the main device.
5. The second pre-polymer solution was added on to the same coverslip where the first unit was cured. The spacer cover was then placed on to the coverslip and the device was exposed to UV light. These steps were repeated until the desired geometry was formed. At the end of this experiment, a prototype of digitally specified patterns was fabricated.

Table 2. 1: The working parameters of 3D gel fabrication

Exposure time	35 s
Height	60 mm
Power	6.25 W/cm^2
PI concentration	1 %
GelMA concentration	5 %
Cell concentration	10^6 cells/ml

2.2.8. Preparation of Cells

Three different cell lines were used for cell experiments. L929 (fibroblast), SH-SY5Y (neuroblastoma), HUVEC (endothelial cells) cells that had been stored in liquid nitrogen were thawed individually at the passage of 33, 16, and 18 respectively and suspended in Dulbecco's Modified Eagle Medium (Biological Industries, Cromwell, CT, USA) which contains 10 % fetal bovine serum (Biological Industries, Cromwell, CT, USA) , 1% penicillin/streptomycin (Gibco, Waltham, MA, USA) and 1% L-glutamine(Gibco, Waltham, MA,USA). Cells were seeded in cell culture dishes and incubated at 37 ºC with a 95% relative humidity, and 5 % CO_2. Cells were sub-cultured twice prior to viability and cell tracker experiments.

2.2.9. Three-dimensional neural co-culture preparation

L929, SH-SY5Y, and HUVEC cells were trypsinized with 0.25 % trypsin-EDTA (Gibco, Waltham, MA, USA) and mixed into the pre-polymer solution at a concentration of 10^6 cells ml^{-1}. 30 µl of the mixture was loaded on to TMSPMA coated coverslip by pipetting and hydrogel posts were cured using UV light for 35 seconds at a power of 6.25 W/cm^2 and the distance of 60 mm. Once the first element was formed, the coverslip was washed with DPBS to remove the un-crosslinked pre-polymer solution. The mask frame was replaced with the second element and the second pre-polymer solution, which consists of another type of cell, was injected on to the same coverslip and cured under UV light. This process was repeated until the desired geometry was completed. Once the desired pattern was completed, the coverslip was transferred into a six-well plate and pattern was washed with complete medium six times with 5 minutes time intervals to remove the cytotoxic photoinitiator. Six-well plate was then placed into the incubator that provides the conditions of 37 °C, 95 % relative humidity and 5 % CO_2. Cell culture medium was refreshed every other day.

2.2.10. Cell viability assay

The viability of three-dimensional tissue constructs was assessed using Live/death viability assay (Biovision, Inc., CA, USA). The live cell staining cell dye (1:1000) and the dead cell staining dye (1:10000) were diluted in assay buffer. The cell encapsulated hydrogel posts were then incubated for 45 minutes at 37°C prior to imaging. The viability assay was performed at day 0, 1, 4 and 7. Fluorescent images were taken via Olympus CKX53 inverted microscope. Live and dead cells were observed at the emission of 530 nm and 635 nm respectively. 5-8 images were taken from the three-dimensional tissue constructs at different focus points and a Z-stack of images was formed using NIH Image J software. The Z-stack was projected into one single image using the pixel intensity summation tool of Image J, further analyzes were then performed. NIH Image J was used to quantify the results and count the cells.

2.2.11. Functional immunofluorescent staining

Distinct cell types were identified using functional immunofluorescent staining using fluorescent cell trackers. In this study, 3-unit and 2-unit patterns were used and these individual elements were encapsulated different cell types. Qtracker^{TM} 655 cell labeling kit 625 cell tracker (red), Qtracker ^{TM} 525 cell labeling kit (green) and CellTracker^{TM} Blue CMF_2HC dye were purchased from Life Technologies, NY, USA. For Qtrackers; component A and B for both 525 and 655 were mixed equal amounts in individual 1.5 ml centrifuge tubes and then incubated for 5 minutes at room temperature. Then diluted (1:200) with complete medium and vortexed 30 seconds. Culture medium was replaced with immunostaining solution and cells were incubated for 24 hours at 37 °C and 5% CO_2. CMF_2HC dye was serum sensitive so; the immunostaining solution was prepared by diluting (1:400) the dye in serum-free medium. Cell culture medium removed and cells were washed serum-free medium to make sure there weren't any serum residues. The immunostaining solution was then added to cell culture dish and cells were incubated for 24 hours at 37 °C and 5% CO_2.

Cells were trypsinized with 0.25 % trypsin-EDTA after 24-hour incubation and mixed with a pre-polymer solution at a concentration of 10^6 cells ml^{-1}. The mixture was loaded on to TMSPMA coated coverslip that stabilized on the alignment device and exposed to UV light for 35 seconds to crosslink the first element of the desired pattern. The un-crosslinked pre-polymer solution was washed away with DPBS and the second pre-polymer solution was loaded on to the same coverslip. The process was repeated until the desired pattern was formed. At the end of the process digitally designed patterns was been able to see as different colored cell-loaded units. The fluorescent images were taken with Olympus CKX53 inverted microscope and images were stacked, projected and analyzed with NIH image J software.

3. RESULTS

3.1. H^1 NMR characterization GelMA

The methacrylation of GelMA was controlled using H NMR technique. The peak at 7.4 ppm was corresponding to the aromatic residues of gelatin. The peaks at 5.59 ppm and 5.80 ppm were corresponding to the methacrylamide groups. The H NMR data proved that the methacrylation of gelatin was successful and gelMA was ready to use for photopolymerization (Figure 3.1 and 3.2).

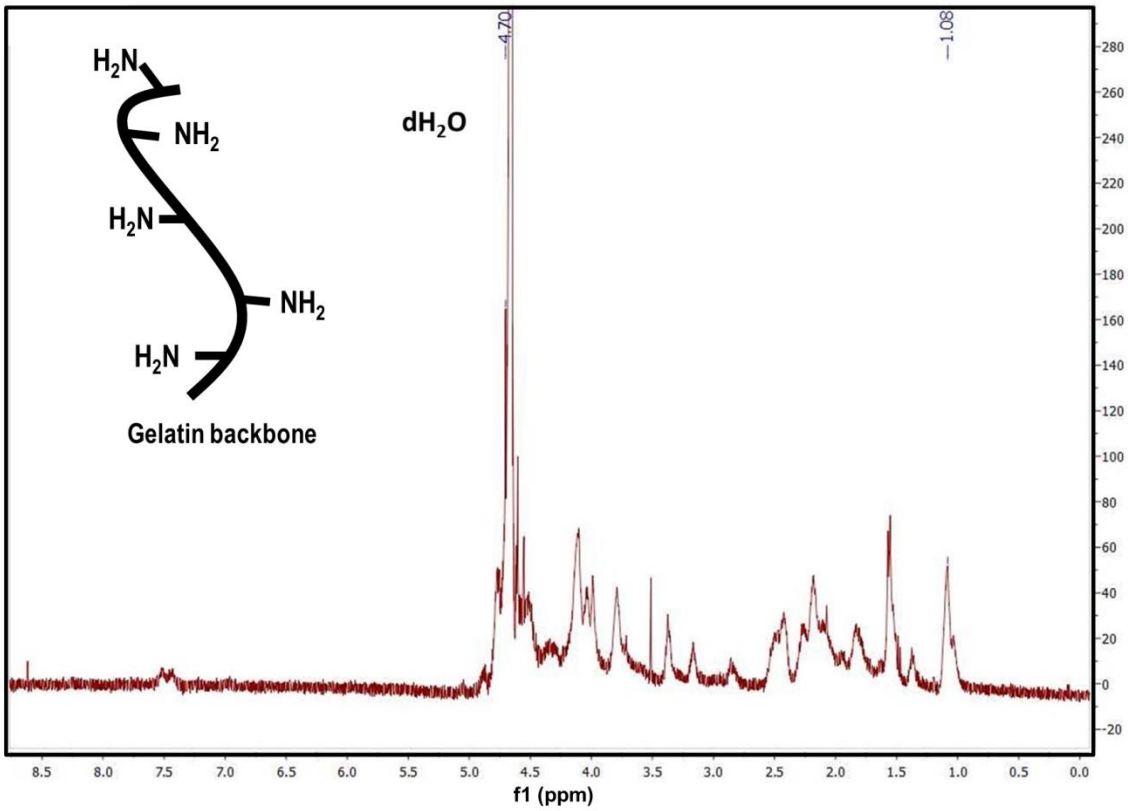

Figure 3. 1: H^1 NMR characterization of gelatin

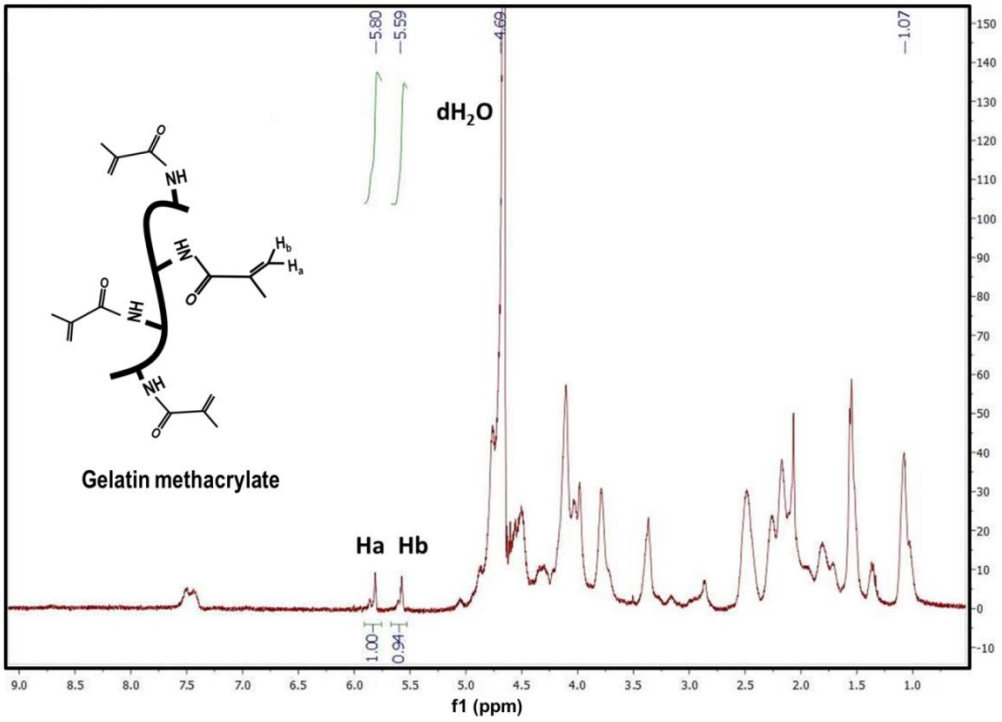

Figure 3. 2: H^1 NMR characterization of gelatin methacrylate

3.2. Characterization of the 3D gel fabrication

The precision of the alignment and the gel fabrication of the device were characterized using colored and transparent hydrogel posts. The gel posts were fabricated with a thickness of 300 µm using three different photomask sets. Every unit of the geometry was fabricated using a different colored dye to see the alignment and the interfaces of the hydrogel posts (Figure 3.4 and 3.5). It was observed that the geometries were fabricated precisely and the analysis of their sizes showed that the device was fabricated the textures accurately. To show the alignment precision, transparent gels were used and after fabricating the whole geometry, a drop of blue dye was injected on the coverslip to see whether the dye will go through between the units of geometry. It was observed that the dye only colored the outside of the hydrogel posts and this proved that there was no space between the hydrogel units (Figure 3.3).

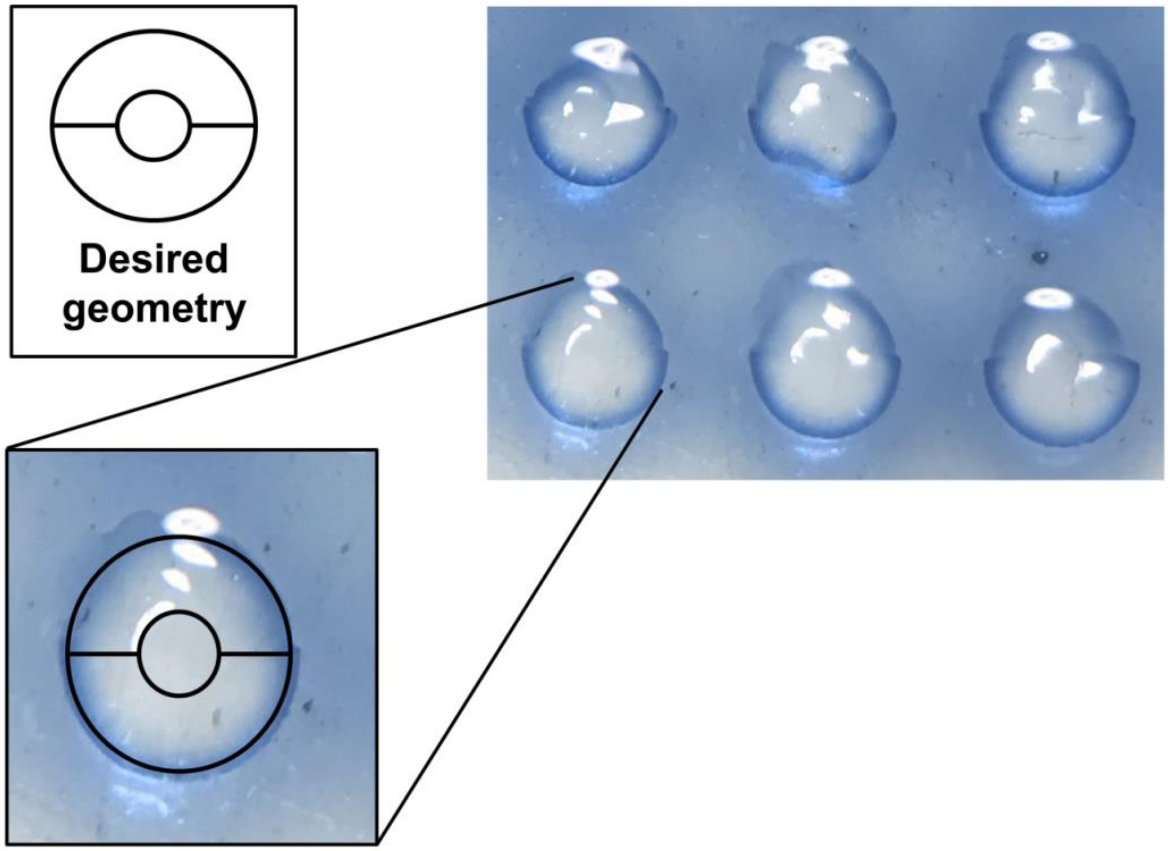

Figure 3. 3: Alignment test for the precision of the fabrication

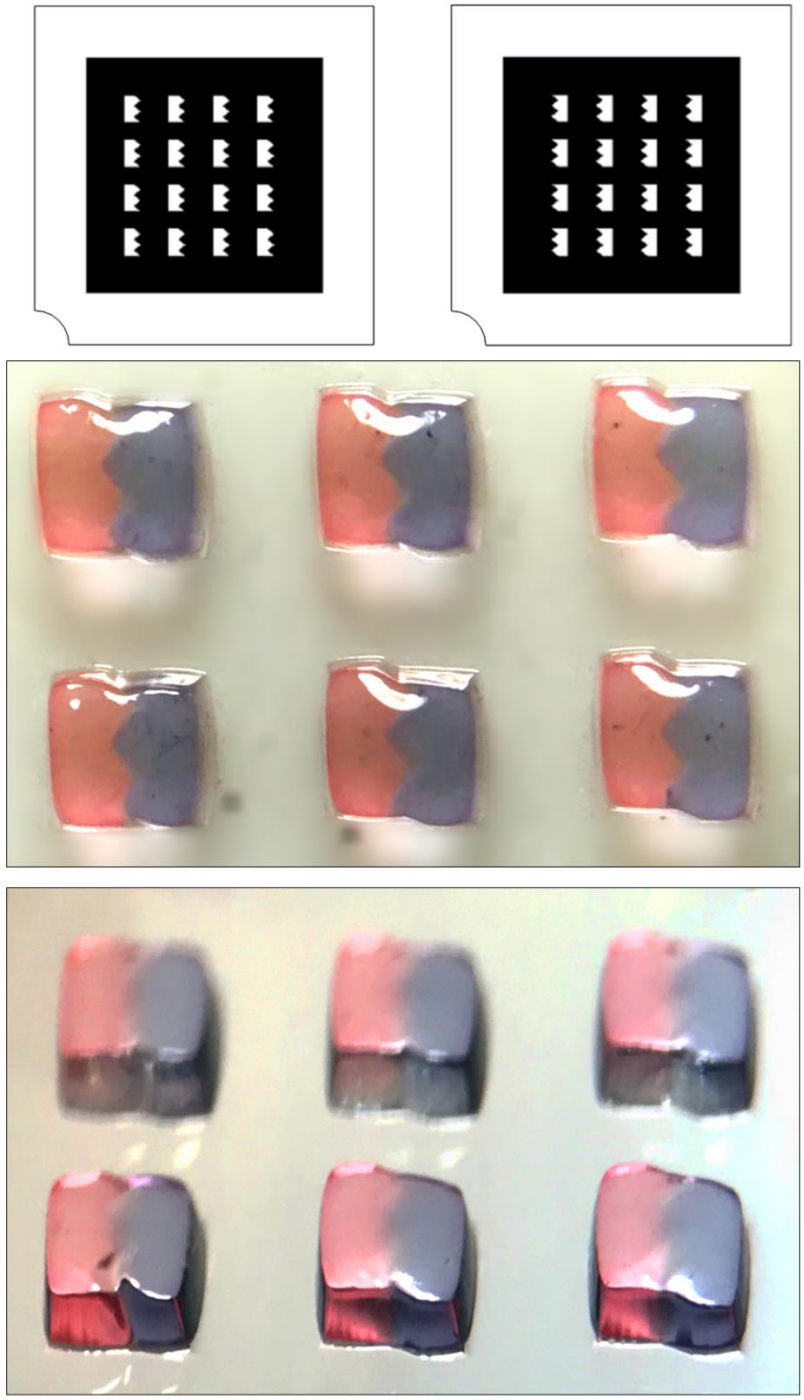

Figure 3. 4: Colored display of saw photomask

Figure 3. 5: Colored display of 120° circle photomask

3.3. Cell Viability Tests

The viability of co-cultured cells was studied. In this study, a proof of concept brain model was studied and neuroblastoma cell line (SH-SY5Y), endothelial cell line (HUVEC) and fibroblast cell line (L929) was used. Two different photomask sets were applied to create co-culturing systems. *Saw* photomasks were used for co-culturing SH-SY5Y and HUVEC cells and two types of cells were co-cultured for 7 days. A fluorescent cell viability kit was used to track the viability of cells. The imaging was performed at the days of 0, 1, 4 and 7, and the viability of the cells that are co-cultured using saw photomasks was 86 %, 80%, 89% and 83%, respectively (n=6). The overall viability was above 80%, which is a promising value for further studies.

The other photomask set was half doughnut photomasks that allow co-culturing three types of cells together. In this set, SH-SY5y, HUVEC, and L929 cells were encapsulated into gelMA units and co-cultured for 7 days. The imaging was performed in the days of 0, 1, 4 and 7, and the viability of the cells was 87%, 82%, 88% and 85 %, respectively (n=6). The overall viability for half doughnut photomask set was above 80 %, as well (Figure 3.6, 3.7 and 3.8).

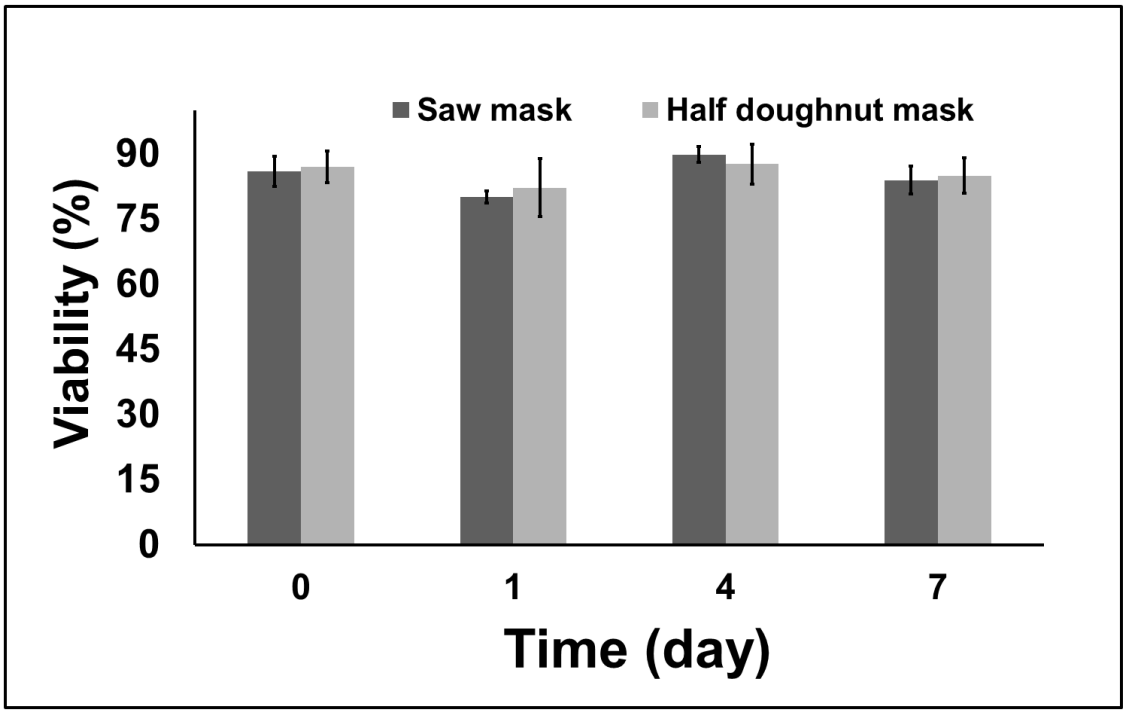

Figure 3. 6: The viability of both saw and half doughnut photomask sets (n=6)

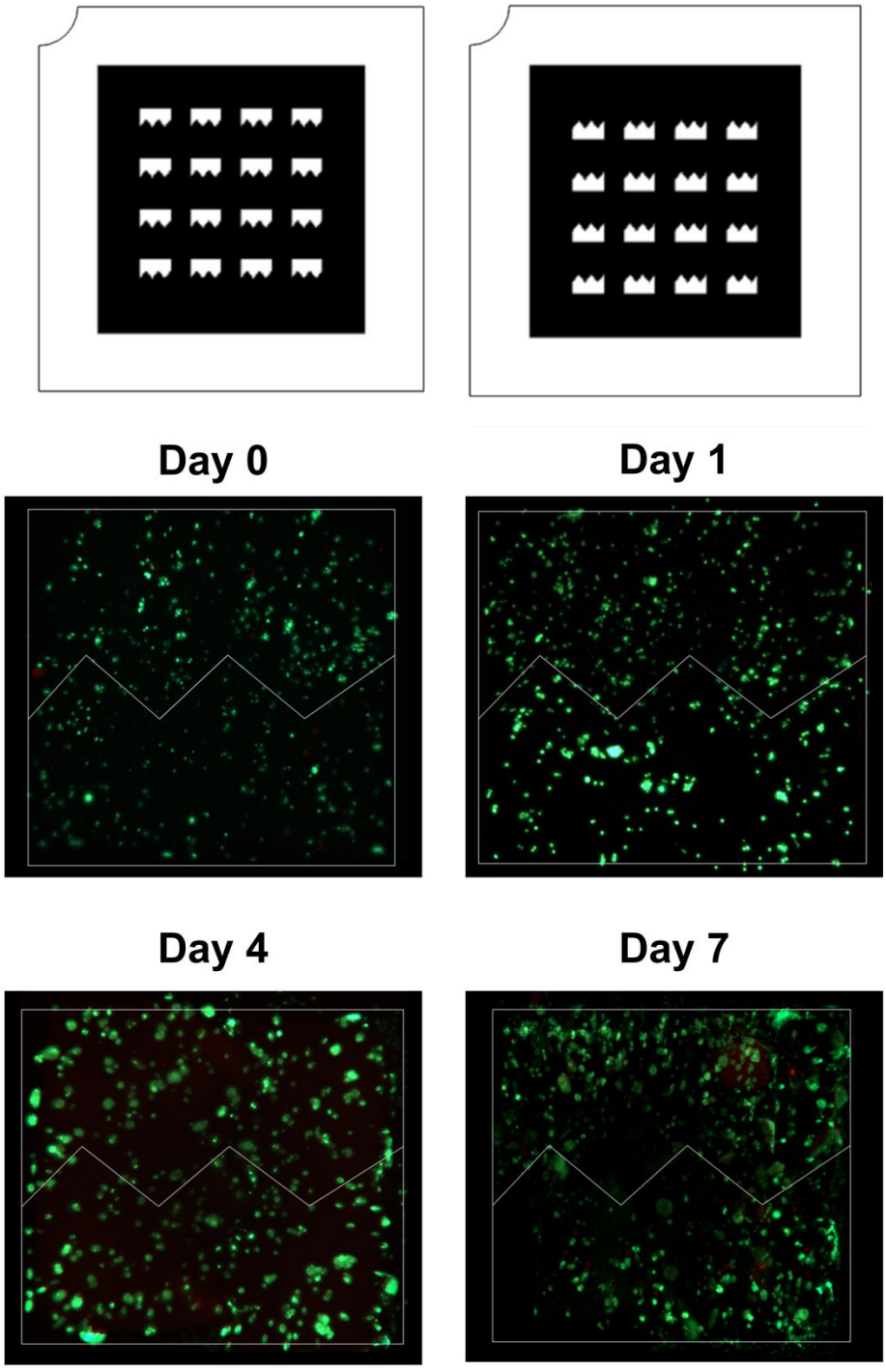

Figure 3. 7: The viability images of saw photomask set

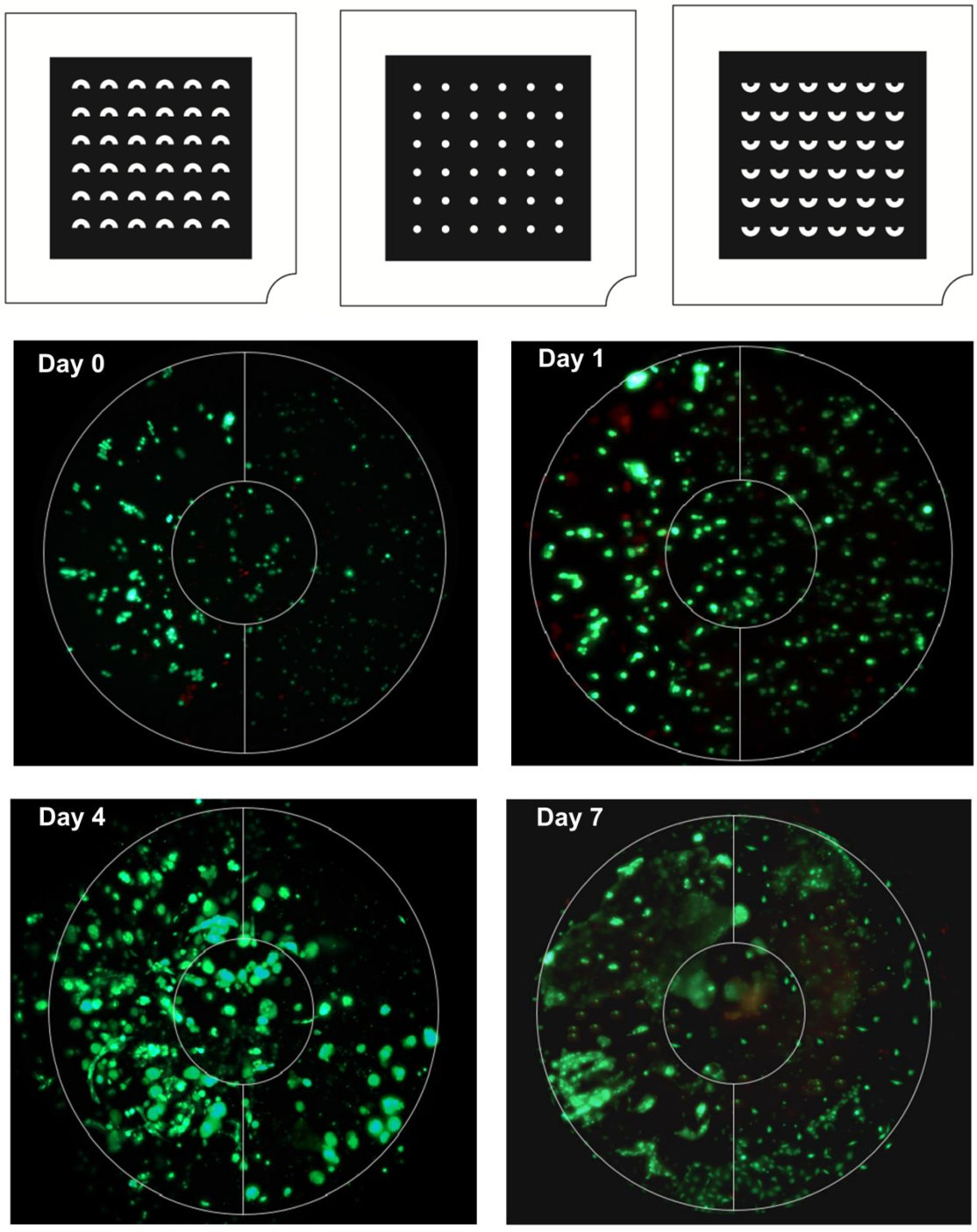

Figure 3. 8: Half doughnut photomask set viability images

3.4. Functional staining of cells

Fluorescent cell trackers were used to staining the different cell types. Qtracker 655 stains cells in red, Qtracker 525 stains cells in green and CellTracker^{TM}Blue CMF_2HC stains cells in blue. In this study SH-SY5Y cells were stained with Qtracker 525, HUVEC cells were stained with CellTracker^{TM}Blue CMF_2HC and L929 cells were stained with Qtracker 655. These immunostains were used to show how cells are located and distributed in distinct gel units of individual geometries as a proof of concept.

Six particularly textured geometries were used to demonstrate the distribution. These shapes were formed using the sets of saw photomask, doughnut photomask, IKC photomask, half doughnut photomask, squares photomask and 120° photomask. The sets of saw, doughnut, and squares photomasks contain two types of cells that are combinations of HUVEC, L929, and SH-SY5Y. On the other hand, the sets of IKC, half doughnut, and 120°photomasks contain all three types of cells (Figure 3.9-3.14).

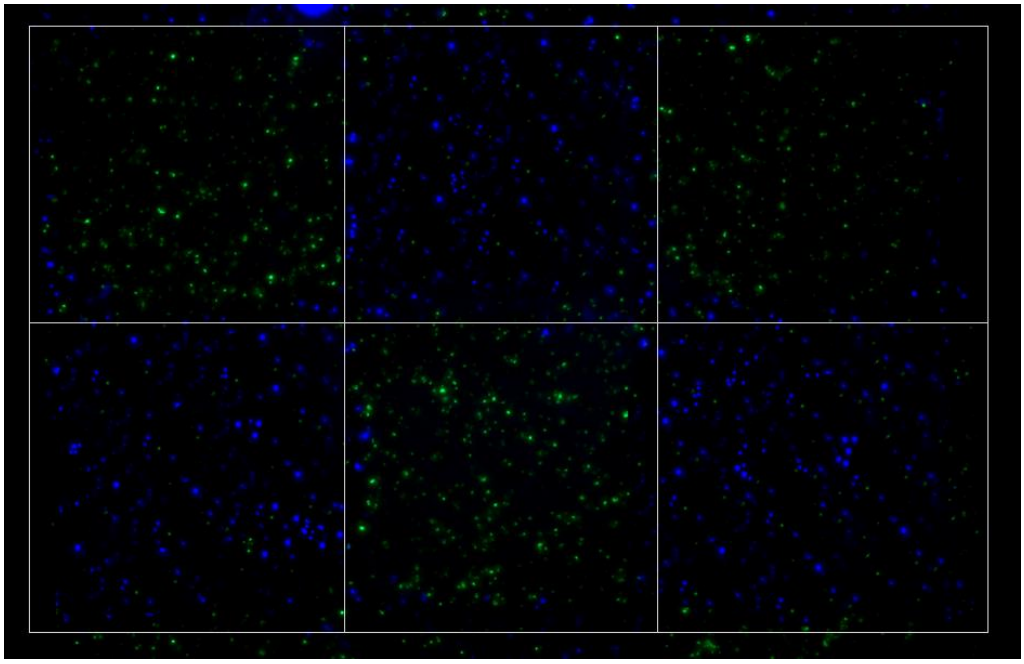

Figure 3. 9:The distribution of HUVEC (blue) and SH-SY5Y (green) in squares photomask set

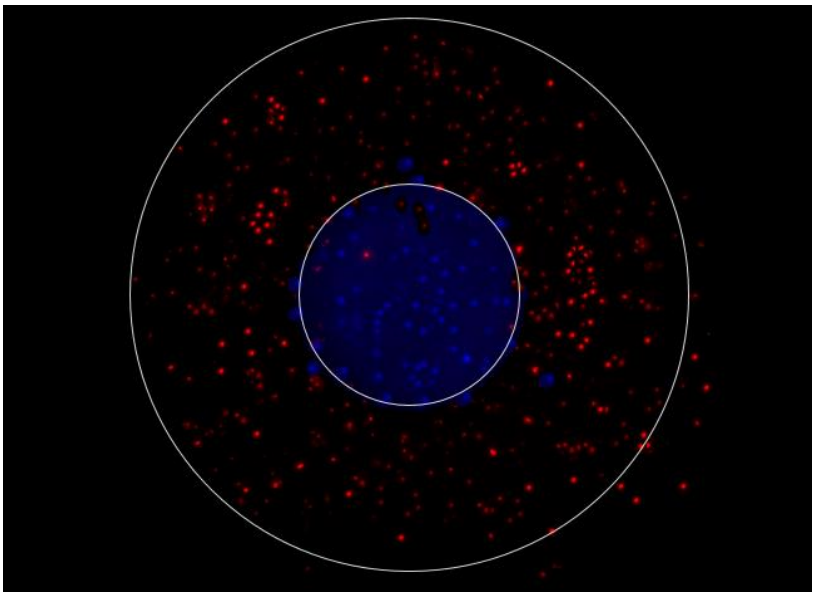

Figure 3. 10: The distribution of HUVEC (blue) and L929 (red) in squares photomask set

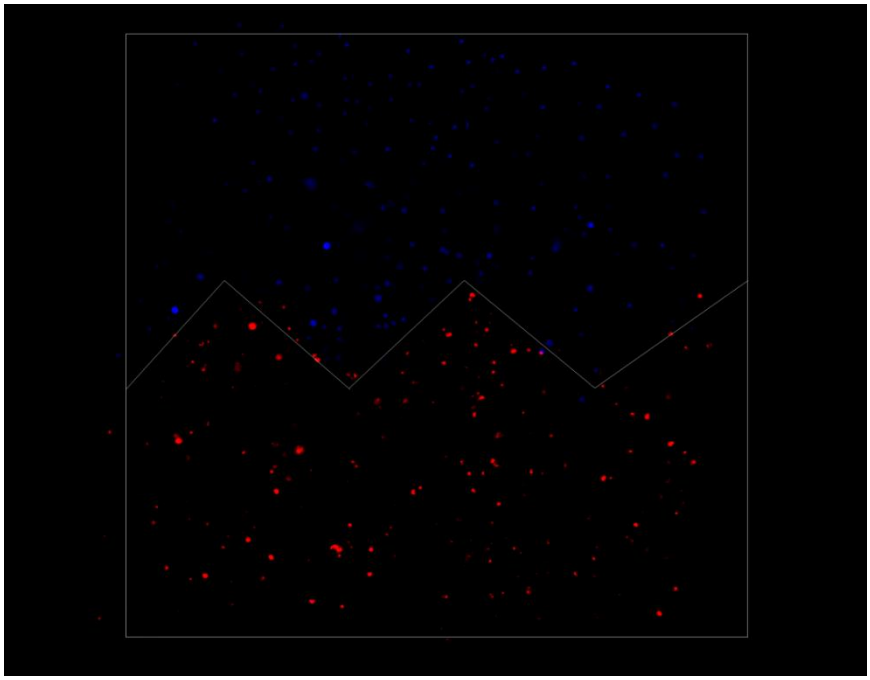

Figure 3. 11: The distribution of HUVEC (blue) and SH-SY5Y (green) in saw photomask set

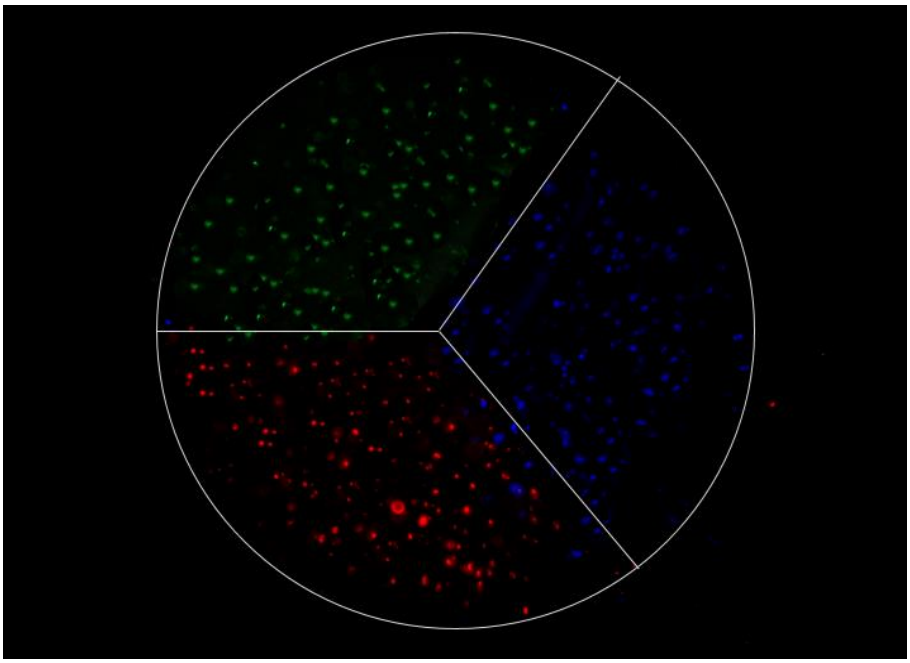

Figure 3. 12: The distribution of HUVEC (blue), L929 (red) and SH-SY5Y (green) in 120° photomask set

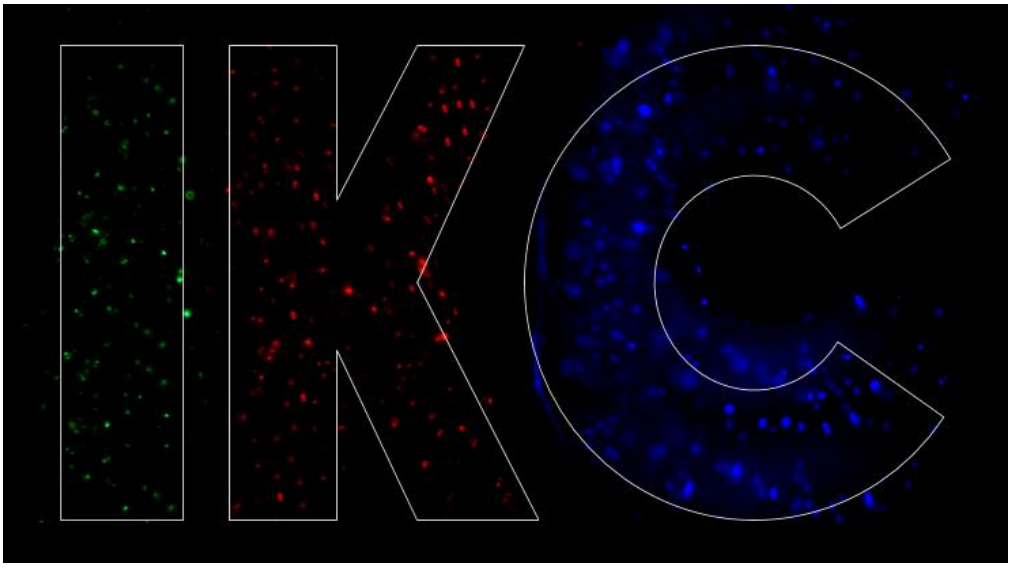

Figure 3. 13: The distribution of HUVEC (blue), L929 (red) and SH-SY5Y (green) in IKC photomask set

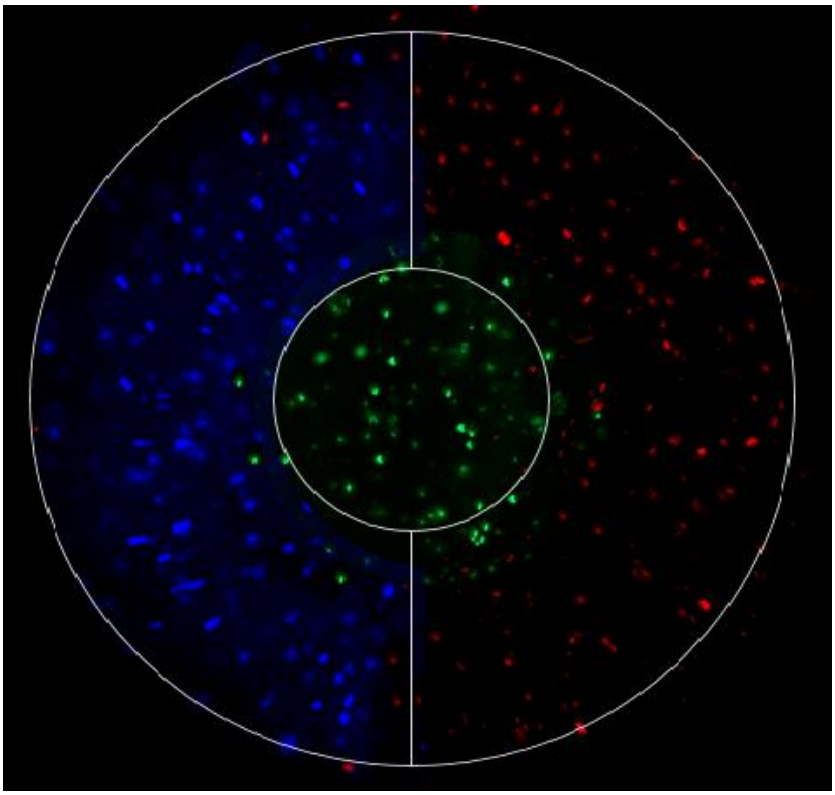

Figure 3. 14: The distribution of HUVEC (blue), L929 (red) and SH-SY5Y (green) in half doughnut photomask set

4. DISCUSSION

Co-culturing different type of cells while obtaining the structure of the extracellular matrix is one of the goals of tissue modeling. One cannot ignore the complex and rich structure of the tissues, while biomimicking the organ construction. In this study neural cells, endothelial cells and epithelial cells were cultured together as a proof of concept modeling for brain structure. It is known that brain has various types of neural cells such as neurons, glial cells, astrocytes, microglia and oligodendrocytes[63]. On the other hand, the blood-brain barrier has endothelial cells and epithelial cells to build a barrier around the brain which develops a selective permeability of the component that is carried via blood[13]. As Three different cells that present in brain structure were cultured, it has been shown that an in vitro model can be created using hydrogel scaffolds and computer-aided designed photomasks. This approach lets scientist design different textures to model the brain and blood-brain barrier architecture and the interfaces between them. Photomasks allow building precise interfaces between different cell types encapsulated hydrogel posts and provide a well-modeled environment for further studies.

4.1. Hydrogel fabrication

The encapsulation of cells into hydrogel posts is a well-explained method for three-dimensional cell culture. There are methods that let scientist create a textured gel post including 3D printing and computer-aided designed photomasks. 3D printing is one of the emerging methods that let scientists create different designed hydrogel posts. However, due to high prices and well-trained people requirements, not all groups are able to use this technology. At this point, the basic photopolymerization methods using either UV or visible light bring a great advantage for lab scaled applications.

Computer aided designed photomasks to allow creating precise geometries without any requirement of well-trained personnel. On the other hand, this approach is way inexpensive compared to 3D printing devices. Even though 3D printers provide a well-controlled environment with the assistance of design software, the fabrication of hydrogel blocks via UV photopolymerization is a cost-effective, precise a practical alternative to this high-tech

devices. The moderate cost of a 3D bioprinter is approximately $ 20.000[59] whereas the cost of our system both including device and UV source is about $ 13.000 (Table 4.1).

Table 4. 1: The cost of hydrogel fabrication system

The cost of gel fabrication by UV photopolymerization	
UV source	$ 12000
Fabrication device	$ 13.12
Each photomask (including frame)	$ 0.52

4.2. Neural tissue modeling

Gurkan et al. (2013) were reported previously that endothelial cells, epithelial cells, and embryonic stem cells can be encapsulated in distinct gel posts and cultured together for long term. Differentiation of stem cells with the presence of the other two types of cells was achieved in this study and also the high viability of cells was reported[22]. Even though brain abundantly contains neural cells (neurons, glial cells, astrocytes etc.), it also has endothelial and epithelial cells in blood-brain barrier, which is a selective barrier that protects the brain. So, a simple, proof of concept creation of neural tissue can be build using neural cells, epithelial cells, and endothelial cells.

There are two approaches which are either encapsulating cells into distinct hydrogel posts or seeding cells on to hydrogel scaffolds sequentially to fabricate a three-dimensional neural tissue model. In the second approach, different cells are seeded on to the scaffold at different time points of the incubation and this brings an adaptation stress on cells at different time points over and over again. However, when cells are encapsulated into hydrogel posts and incubated at the same time, their adaptation period begins at the same time and no gradual adaptation stress occurs on the cells. From this point, encapsulating cells using UV photopolymerization method become an advantageous approach to keep cells less stressed while allowing them to adapt each other’s presence in vitro. Considering this phenomenon,

neuroblastoma cell line (SH-SY5Y), endothelial cell line(HUVEC) and epithelial cell line (L929) were encapsulated in separate hydrogel posts which were in touch with each other and in this way, both tissue model and the precision of fabrication device were tested and positive results were achieved.

4.3. Fabrication device

A neural tissue model must have different cell types together and hence for being able to create an appropriate model, co-culturing cells is essential. While co-culturing cells, which are encapsulated in separate hydrogel posts, the connection between cells must be precisely ensured, hereby a device that will allow the user to create precise geometries was required. Although, 3D printing technologies fabricate perfectly precise geometries with pretty high resolutions, an affordable and easy to get alternative is still needed for lab scale applications.

Gurkan et al. (2013) have used a sandwich-like device to construct their hydrogel posts, yet their design was not practical enough for further studies. In this study, we designed a drawer-like device and limit the mobile parts as much as possible to make precision higher. The more precise fabrication will let us achieve more connected hydrogel posts which means the more connected cells.

5. CONCLUSION

Neural tissue modeling is high in demand for further studies since it is hard to work on animal models due to both ethical issues and the challenges to understand what really happens in the signaling pathways in the complex structure of the brain. At this point, a simple model that will allow scientists to understand how a magnificent network of the neurons forms carries a great importance. Co-culturing the cells that present in the structure of the brain is the first step of in vitro neural tissue modeling prior to creating a fully functioning model achievement. On the other hand, culturing cells in a three-dimensional environment is essential to mimic the extracellular matrix and obtain veracious tissue models. For this purpose, we designed a novel three-dimensional hydrogel co-culturing system. This system requires a light which can be either UV light or visible light that will trigger the photopolymerization process. Also, this device lets users create various geometrical textures using computer-aided designed photomasks. Since the main aim of this study is modeling a neural tissue which is a well proportionally created with different cell types, the conversation between cells is crucial to understanding the further mechanisms of the tissue composition. Hence, this device allows users build precise and controlled geometries that have an extensive importance for the communication between cells during co-culturing.

The outcomes of the study showed that our fabrication device has promising results and 7-day viability tests proved that our device has nontoxic effect on the cells during fabrication. As an affordable alternative to 3D printing technology, our device will be a good contribution to the three-dimensional tissue culturing field. On the other hand, the high viability of cells indicated that both the photopolymerization technique and the device itself is a considerably promising method for further modeling research.

6. FUTURE PROSPECTS

In this study, we only focused on designing a device that is both easy to use and affordable for lab scale applications and proved that our device works considerably well. So, we can use this device for any kind of tissue modeling such as vascular tissues, tissue interface modelings, tissue junction modelings as well as disease modelings.

We have used quite simple geometries; however complex structures with complex additives in distinct hydrogel posts can be studied for curative approaches, as well. On the other hand, the tissue models that are created with this device can be used for pharmaceutical applications such as drug test or cosmetic tests that cannot be done due to ethical issues.

7. REFERENCES

[1] **An, Y., & Hubbell, J. A.** (2000). Intraarterial protein delivery via intimally-adherent bilayer hydrogels. *Journal of Controlled Release, 64*(1-3), 205-215.

[2] **Augst, A. D., Kong, H. J., & Mooney, D. J.** (2006). Alginate hydrogels as biomaterials. *Macromolecular bioscience, 6*(8), 623-633.

[3] **Bigi, A., Cojazzi, G., Panzavolta, S., Rubini, K., & Roveri, N.** (2001). Mechanical and thermal properties of gelatin films at different degrees of glutaraldehyde crosslinking. *Biomaterials, 22*(8), 763-768.

[4] **Boyan, B. D., Hummert, T. W., Dean, D. D., & Schwartz, Z.** (1996). Role of material surfaces in regulating bone and cartilage cell response. *Biomaterials, 17*(2), 137-146.

[5] **Bromberg, L. E., & Ron, E. S.** (1998). Temperature-responsive gels and thermogelling polymer matrices for protein and peptide delivery. *Advanced drug delivery reviews, 31*(3), 197-221.

[6] **Bryant, S. J., Nuttelman, C. R., & Anseth, K. S.** (2000). Cytocompatibility of UV and visible light photoinitiating systems on cultured NIH/3T3 fibroblasts in vitro. *Journal of Biomaterials Science, Polymer Edition, 11*(5), 439-457.

[7] **Chowdhury, S. M., & Hubbell, J. A.** (1996). Adhesion prevention with ancrod released via a tissue-adherent hydrogel. *Journal of Surgical Research, 61*(1), 58-64.

[8] **Cohen, S., Baño, M. C., Cima, L. G., Allcock, H. R., Vacanti, J. P., Vacanti, C. A., & Langer, R.** (1993). Design of synthetic polymeric structures for cell transplantation and tissue engineering. *Clinical materials, 13*(1), 3-10.

[9] **Cruise, G. M., Hegre, O. D., Lamberti, F. V., Hager, S. R., Hill, R., Scharp, D. S., & Hubbell, J. A.** (1999). In Vitro and In Vivo Performance of Porcine Islets Encapsulated in Interfacially Photopolymerized PoIy (Ethylene Glycol) Diacrylate Membranes. *Cell transplantation, 8*(3), 293-306.

[10] **Cruise, G. M., Scharp, D. S., & Hubbell, J. A.** (1998). Characterization of permeability and network structure of interfacially photopolymerized poly (ethylene glycol) diacrylate hydrogels. *Biomaterials, 19*(14), 1287-1294.

[11] **Dang, Q. F., Yan, J. Q., Li, J. J., Cheng, X. J., Liu, C. S., & Chen, X. G.** (2011). Controlled gelation temperature, pore diameter and degradation of a highly porous chitosan-based hydrogel. *Carbohydrate Polymers, 83*(1), 171-178. doi:https://doi.org/10.1016/j.carbpol.2010.07.038

[12] **DuRaine, G. D., Brown, W. E., Hu, J. C., & Athanasiou, K. A.** (2015). Emergence of scaffold-free approaches for tissue engineering musculoskeletal cartilages. *Annals of biomedical engineering, 43*(3), 543-554.

[13] **Duus, P.** (1998). *Topical diagnosis in neurology: anatomy, physiology, signs, symptoms*: Thieme Georg Verlag.

[14] **Elisseeff, J., Anseth, K., Sims, D., McIntosh, W., Randolph, M., & Langer, R.** (1999). Transdermal photopolymerization for minimally invasive implantation. *Proceedings of the National Academy of Sciences, 96*(6), 3104-3107.

[15] **Elisseeff, J., McIntosh, W., Anseth, K., Riley, S., Ragan, P., & Langer, R.** (2000). Photoencapsulation of chondrocytes in poly (ethylene oxide)-based semi-interpenetrating networks. *Journal of Biomedical Materials Research Part A, 51*(2), 164-171.

[16] **Fairbanks, B. D., Schwartz, M. P., Bowman, C. N., & Anseth, K. S.** (2009). Photoinitiated polymerization of PEG-diacrylate with lithium phenyl-2, 4, 6-trimethylbenzoylphosphinate: polymerization rate and cytocompatibility. *Biomaterials, 30*(35), 6702-6707.

[17] **Fleming, M. G., & Maillet, W. A.** (1999). Photopolymerization of composite resin using the argon laser. *Journal-Canadian Dental Association, 65*, 447-452.

[18] **Fouassier, J.-P.** (1995). *Photoinitiation, photopolymerization, and photocuring: fundamentals and applications*: Hanser.

[19] **Fouassier, J. P., Burr, D., & Wieder, F.** (1991). Water-soluble photoinitiators: Primary processes in hydroxy alkyl phenyl ketones. *Journal of Polymer Science Part A: Polymer Chemistry*, *29*(9), 1319-1327.

[20] **Frantz, C., Stewart, K. M., & Weaver, V. M.** (2010). The extracellular matrix at a glance. *Journal of Cell Science, 123*(24), 4195-4200. doi:10.1242/jcs.023820

[21] **Gunatillake, P. A., & Adhikari, R.** (2003). Biodegradable synthetic polymers for tissue engineering. *Eur Cell Mater, 5*(1), 1-16.

[22] **Gurkan, U. A., Fan, Y., Xu, F., Erkmen, B., Urkac, E. S., Parlakgul, G., . . . Demirci, U.** (2013). Simple precision creation of digitally specified, spatially heterogeneous, engineered tissue architectures. *Advanced materials, 25*(8), 1192-1198.

[23] **Heijkants, R., Van Tienen, T., De Groot, J., Pennings, A., Buma, P., Veth, R., & Schouten, A.** (2006). Preparation of a polyurethane scaffold for tissue engineering made by a combination of salt leaching and freeze-drying of dioxane. *Journal of materials science, 41*(8), 2423-2428.

[24] **Hench, L. L.** (1991). Bioceramics: from concept to clinic. *Journal of the american ceramic society, 74*(7), 1487-1510.

[25] **Hench, L. L.** (1998). Biomaterials: a forecast for the future. *Biomaterials, 19*(16), 1419-1423. doi:https://doi.org/10.1016/S0142-9612(98)00133-1

[26] **Herning, T., Djabourov, M., Leblond, J., & Takerkart, G.** (1991). Conformation of gelatin chains in aqueous solutions: 2. A quasi-elastic light scattering study. *Polymer, 32*(17), 3211-3217.

[27] **Hill-West, J. L., Chowdhury, S. M., Dunn, R. C., & Hubbell, J. A.** (1994). Efficacy of a resorbable hydrogel barrier, oxidized regenerated cellulose, and hyaluronic acid in the prevention of ovarian adhesions in a rabbit model. *Fertility and sterility, 62*(3), 630-634.

[28] **Hoch, E., Schuh, C., Hirth, T., Tovar, G. E., & Borchers, K.** (2012). Stiff gelatin hydrogels can be photo-chemically synthesized from low viscous gelatin solutions using molecularly functionalized gelatin with a high degree of methacrylation. *Journal of Materials Science: Materials in Medicine, 23*(11), 2607-2617.

[29] **Hutmacher, D. W., & Singh, H.** (2008). Computational fluid dynamics for improved bioreactor design and 3D culture. *TRENDS in Biotechnology*, *26*(4), 166-172.

[30] **Jhon, M. S., & Andrade, J. D.** (1973). Water and hydrogels. *Journal of Biomedical Materials Research Part A, 7*(6), 509-522.

[31] **Jones, C. A., Huberman, E., Cunningham, M. L., & Peak, M. J.** (1987). Mutagenesis and cytotoxicity in human epithelial cells by far-and near-ultraviolet radiations: action spectra. *Radiation research*, *110*(2), 244-254.

[32] **Kang, H.-W., Tabata, Y., & Ikada, Y.** (1999). Fabrication of porous gelatin scaffolds for tissue engineering. *Biomaterials*, *20*(14), 1339-1344.

[33] **Kappes, U. P., Luo, D., Potter, M., Schulmeister, K., & Rünger, T. M.** (2006). Short-and long-wave UV light (UVB and UVA) induce similar mutations in human skin cells. *Journal of Investigative Dermatology*, *126*(3), 667-675.

[34] **Kim, I.-S., Jeong, Y.-I., & Kim, S.-H.** (2000). Self-assembled hydrogel nanoparticles composed of dextran and poly (ethylene glycol) macromer. *International journal of pharmaceutics*, *205*(1-2), 109-116.

[35] **Kirkpatrick, C. J., Fuchs, S., & Unger, R. E.** (2011). Co-culture systems for vascularization—learning from nature. *Advanced drug delivery reviews*, *63*(4-5), 291-299.

[36] **Lee, K.-W., Wang, S., Fox, B. C., Ritman, E. L., Yaszemski, M. J., & Lu, L.** (2007). Poly (propylene fumarate) bone tissue engineering scaffold fabrication using stereolithography: effects of resin formulations and laser parameters. *Biomacromolecules, 8*(4), 1077-1084.

[37] **Lee, K. Y., Bouhadir, K. H., & Mooney, D. J.** (2000). Degradation behavior of covalently cross-linked poly (aldehyde guluronate) hydrogels. *Macromolecules, 33*(1), 97-101.

[38] **Lee, K. Y., & Mooney, D. J.** (2001). Hydrogels for tissue engineering. *Chemical reviews, 101*(7), 1869-1880.

[39] **Lee, S. B., Kim, Y. H., Chong, M. S., Hong, S. H., & Lee, Y. M.** (2005). Study of gelatin-containing artificial skin V: fabrication of gelatin scaffolds using a salt-leaching method. *Biomaterials, 26*(14), 1961-1968.

[40] **Lien, S.-M., Ko, L.-Y., & Huang, T.-J.** (2009). Effect of pore size on ECM secretion and cell growth in gelatin scaffold for articular cartilage tissue engineering. *Acta biomaterialia, 5*(2), 670-679.

[41] **Lin, H., Zhang, D., Alexander, P. G., Yang, G., Tan, J., Cheng, A. W.-M., & Tuan, R. S.** (2013). Application of visible light-based projection stereolithography for live cell-scaffold fabrication with designed architecture. *Biomaterials, 34*(2), 331-339. doi:https://doi.org/10.1016/j.biomaterials.2012.09.048

[42] **Lo, H., Ponticiello, M., & Leong, K.** (1995). Fabrication of controlled release biodegradable foams by phase separation. *Tissue engineering, 1*(1), 15-28.

[43] **Lopérgolo, L. C., Lugao, A. B., & Catalani, L. H.** (2003). Direct UV photocrosslinking of poly (N-vinyl-2-pyrrolidone)(PVP) to produce hydrogels. *Polymer, 44*(20), 6217-6222.

[44] **Maffezzoli, A., Pietra, A. D., Rengo, S., Nicolais, L., & Valletta, G.** (1994). Photopolymerization of dental composite matrices. *Biomaterials, 15*(15), 1221-1228.

[45] **Mann, B. K., Gobin, A. S., Tsai, A. T., Schmedlen, R. H., & West, J. L.** (2001). Smooth muscle cell growth in photopolymerized hydrogels with cell adhesive and proteolytically degradable domains: synthetic ECM analogs for tissue engineering. *Biomaterials, 22*(22), 3045-3051.

[46] **Mironi-Harpaz, I., Wang, D. Y., Venkatraman, S., & Seliktar, D.** (2012). Photopolymerization of cell-encapsulating hydrogels: crosslinking efficiency versus cytotoxicity. *Acta biomaterialia, 8*(5), 1838-1848.

[47] **Nguyen, K. T., & West, J. L.** (2002). Photopolymerizable hydrogels for tissue engineering applications. *Biomaterials, 23*(22), 4307-4314. doi:https://doi.org/10.1016/S0142-9612(02)00175-8

[48] **Nichol, J. W., Koshy, S. T., Bae, H., Hwang, C. M., Yamanlar, S., & Khademhosseini, A.** (2010). Cell-laden microengineered gelatin methacrylate hydrogels. *Biomaterials, 31*(21), 5536-5544.

[49] **Nicodemus, G. D., & Bryant, S. J.** (2008). Cell encapsulation in biodegradable hydrogels for tissue engineering applications. *Tissue Engineering Part B: Reviews, 14*(2), 149-165.

[50] **Pathak, C. P., Sawhney, A. S., & Hubbell, J. A.** (1992). Rapid photopolymerization of immunoprotective gels in contact with cells and tissue. *Journal of the American Chemical Society, 114*(21), 8311-8312.

[51] **Ratner, B. D., Hoffman, A. S., Schoen, F. J., & Lemons, J. E.** (2004). *Biomaterials science: an introduction to materials in medicine*: Academic press.

[52] **Reece, J. B., Urry, L. A., Cain, M. L., Wasserman, S. A., Minorsky, P. V., & Jackson, R. B.** (2011). *Campbell biology* (Vol. 9): Pearson Boston.

[53] **Rezwan, K., Chen, Q., Blaker, J., & Boccaccini, A. R.** (2006). Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. *Biomaterials, 27*(18), 3413-3431.

[54] **Rosiak, J., Olejniczak, J., & Pękala, W.** (1990). Fast reaction of irradiated polymers—I. Crosslinking and degradation of polyvinylpyrrolidone. *International Journal of Radiation Applications and Instrumentation. Part C. Radiation Physics and Chemistry, 36*(6), 747-755.

[55] **Rosiak, J., Rucinska-Rybus, A., & Pekala, W.** (1989). Method of manufacturing hydrogel dressings: Google Patents.

[56] **Sawhney, A. S., Pathak, C. P., & Hubbell, J. A.** (1993). Interfacial photopolymerization of poly (ethylene glycol)-based hydrogels upon alginate-poly (l-lysine) microcapsules for enhanced biocompatibility. *Biomaterials, 14*(13), 1008-1016.

[57] **Sawhney, A. S., Pathak, C. P., van Rensburg, J. J., Dunn, R. C., & Hubbell, J. A.** (1994). Optimization of photopolymerized bioerodible hydrogel properties for adhesion prevention. *Journal of Biomedical Materials Research Part A, 28*(7), 831-838.

[58] **Scranton, A. B., Bowman, C. N., & Peiffer, R. W.** (1997). *Photopolymerization: fundamentals and applications*: ACS Publications.

[59] **Sher, D.** (2015). THE TOP 15 BIOPRINTERS. Retrieved from https://3dprintingindustry.com/news/top-10-bioprinters-55699/

[60] **Suggs, L. J., & Mikos, A. G.** (1999). Development of poly (propylene fumarate-co-ethylene glycol) as an injectable carrier for endothelial cells. *Cell transplantation, 8*(4), 345-350.

[61] **Taboas, J., Maddox, R., Krebsbach, P., & Hollister, S.** (2003). Indirect solid free form fabrication of local and global porous, biomimetic and composite 3D polymer-ceramic scaffolds. *Biomaterials, 24*(1), 181-194.

[62] **Thomson, R., Wake, M., Yaszemski, M. J., & Mikos, A.** (1995). Biodegradable polymer scaffolds to regenerate organs *Biopolymers Ii* (pp. 245-274): Springer.

[63] **Urenjak, J., Williams, S. R., Gadian, D. G., & Noble, M.** (1993). Proton nuclear magnetic resonance spectroscopy unambiguously identifies different neural cell types. *Journal of Neuroscience, 13*(3), 981-989.

[64] **Van Den Bulcke, A. I., Bogdanov, B., De Rooze, N., Schacht, E. H., Cornelissen, M., & Berghmans, H.** (2000). Structural and rheological properties of methacrylamide modified gelatin hydrogels. *Biomacromolecules, 1*(1), 31-38.

[65] **West, J. L., & Hubbell, J. A.** (1996). Separation of the arterial wall from blood contact using hydrogel barriers reduces intimal thickening after balloon injury in the rat: the roles of medial and luminal factors in arterial healing. *Proceedings of the National Academy of Sciences, 93*(23), 13188-13193.

[66] **Wong, W. H., & Mooney, D. J.** (1997). Synthesis and properties of biodegradable polymers used as synthetic matrices for tissue engineering *Synthetic biodegradable polymer scaffolds* (pp. 51-82): Springer.

[67] **Yoon, H. J., Shin, S. R., Cha, J. M., Lee, S.-H., Kim, J.-H., Do, J. T., . . . Bae, H.** (2016). Cold water fish gelatin methacryloyl hydrogel for tissue engineering application. *PloS one*, *11*(10), e0163902.

[68] **Yoon, J. J., Kim, J. H., & Park, T. G.** (2003). Dexamethasone-releasing biodegradable polymer scaffolds fabricated by a gas-foaming/salt-leaching method. *Biomaterials, 24*(13), 2323-2329.

[69] **Yoshimoto, H., Shin, Y., Terai, H., & Vacanti, J.** (2003). A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering. *Biomaterials, 24*(12), 2077-2082.

[70] **Yue, K., Trujillo-de Santiago, G., Alvarez, M. M., Tamayol, A., Annabi, N., & Khademhosseini, A.** (2015). Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels. *Biomaterials*, *73*, 254-271.

CURRICULUM VITAE

Name-Surname	: Fulya ERSOY
Date of Birth	: 19.02.1990
Nationality	: Turkish

Education

Bachelor's Degree- 2009-2014	Ege University Faculty of Engineering Department of Bioengineering
High School- 2004-2008	Adana IMKB Anatolian Teacher's Training High School