Z. L. CÜCÜK

IZMIR KATIP CELEBI UNIVERSITY

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GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

THREE DIMENSIONAL NEUROVASCULAR CO-CULTURE INSIDE A MICROFLUIDIC INVASION CHEMOTAXIS CHIP

M.Sc. THESIS

Zeynel Levent CÜCÜK

Department of Nanoscience and Nanotechnology

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Thesis Advisor: Dr. Şadiye Emel SOKULLU

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İZMİR KATİP ÇELEBİ ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

MİKROAKIŞKAN İNVAZYON KEMOTAKSİS ÇİPTE ÜÇ BOYUTLU NÖROVASKÜLER KO-KÜLTÜR ÇALIŞMALARI

YÜKSEK LİSANS TEZİ

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Zeynel Levent Cücük an **M.Sc.** student of **IKCU Graduate school Of Natural and Applied Sciences** successfully defended the thesis titled **"THREE DIMENSIONAL NEUROVASCULAR CO-CULTURE INSIDE A MICROFLUIDIC INVASION CHEMOTAXIS CHIP",** which he prepared after fulfilling the requirements specified in the associated legislation, before the jury whose signatures are below.

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Date of Submission : 28. 11. 2019
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To my family,

FOREWORD

I would like to thank Izmir Katip Celebi University, Department of Scientific Research Projects for funding this research. Also, I am thankful to Izmir Biomedicine and Genome Center, Field Emission Scanning Electron Microscopy section for their guidance during the imaging process.

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November 2019 Z. Levent CÜCÜK

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ABBREVATIONS

2D: Two Dimensional
3D: Three Dimensional
ALS: Amyotrophic lateral sclerosis
CAD: Computer Aided Design
DPBS: Dulbecco's phosphate buffer saline
EBL: Electron Beam Lithography
ECM: Extracellular Matrix
EUV: Extreme Ultraviolet Lithography
FESEM: Field Emission Scanning Electron Microscopy
FITR: Fourier Transform Infrared Spectroscopy
FN: Fibronectin
GelMA: Gelatin methacrylate
GF: Growth Factor
HUVEC: Human umbilical vein endothelial cells
IC-Chip: Invasion Chemotaxis Chip
IBG: Izmir Biomedicine and Genome Center
LED: Light emitting diode
MA: Methacrylic Anhydride
MSC: Microbiological Safety Cabinet
NIL: Nanoimprint Lithography
NMR: Nuclear Magnetic Resonance
PDMS: Polydimethylsiloxane
PI: Photoinitiator
PI: Propidium Iodide
TE: Tissue Engineering
SEM: Scanning Electron Microscope
USA: United States of America
UV: Ultraviolet
W: Watt

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THREE DIMENSIONAL NEUROVASCULAR CO-CULTURE INSIDE A MICROFLUIDIC INVASION CHEMOTAXIS CHIP

ABSTRACT

One of the most crucial properties of an artificial tissue is it's ability to mimic the natural tissue. For this reason, similarity between the artificial extracellular matrix (ECM) and the natural ECM is highly important. There are many different strategies to create a realistic artificial extracellular matrix. One of those strategies is building hydrogel structures. Building tissues with using hydrogels enables us to use a platform to examine cellular behaviors under different conditions.

Microfluidic chips are becoming a key tools at nanotechnology as with the increase of alternative production techniques and improved capacity of manufacturing devices, it became possible to create microfluidic chips easily with high resolution. In this research, a microfluidic chip was used to create a three dimensional cell culture environment, and to create this extracellular environment, Gelatin Methacrylate (GelMA) was synthesized. With using ultraviolet light and a photoinitiator, a three dimensional hydrogel structure was built. With this method, an alternative and easier strategy was proposed to create a three dimensional environment for co-culture studies inside of microfluidic chips.

Chemical compounds of synthesized GelMA was identified with Fourier transform infrared spectroscopy (FITR), surface morphology was observed with field emission electron microscopy (FESEM), and structural properties was analyzed with atomic force microscopy (AFM). Inside of the produced GelMA hydrogel structure, a neurovascular co-culture was created, and cellular viability inside of the hydrogel created inside of microfluidic chip was compared with the hydrogel created outside of the microfluidic chip. Swelling performance of the hydrogel inside of microfluidic chip was observed visually and porosity of this hydrogel was examined for 72 hours by tracking cellular localization with using immunofluorescence as a proof of concept.

This study showed that GelMA has desired biomechanical properties with offering more than 80% cellular viability for seven days in both platforms. In addition, the results shown that even GelMA was built inside of a microfluidic chip using photopolymerization, it is still a viable platform for 3D cell culture studies while being structurally stable for long durations.

MİKROAKIŞKAN İNVAZYON KEMOTAKSİS ÇİPTE ÜÇ BOYUTLU NÖROVASKÜLER KO-KÜLTÜR ÇALIŞMALARI

ÖZET

Yapay bir dokunun en önemli özelliklerinden biri gerçek dokuyu ne kadar başarılı taklit edebildiğidir. Bu bağlamda oluşturulan ekstrasellüler matrisin, gerçek ekstrasellüler matrise benzerliği büyük önem taşımaktadır. İyi bir yapay ekstrasellüler matris oluşturmak için farklı stratejiler bulunmaktadır. Bunlardan bir tanesi de hidrojel yapılar oluşturmaktır. Hidrojeller kullanılarak oluşturulan bu doku modelleri farklı etkiler altında hücre davranışları incelememiz için önemli bir platform sağlar.

Alternatif üretim tekniklerinin çoğalması ve kullanılan cihazların kalitesinin artması sayesinde çabuk üretilebilen, yüksek çözünürlüklü mikroakışkan çipler nanoteknolojinin önemli araçlarından biri haline gelmiştir. Bu araştırmada üç boyutlu hücre kültürü ortamı oluşturmak için bir mikroakışkan çip kullanılmıştır. Üç boyutlu iskele yapısını oluşturmak için Jelatin Metakrilat (GelMA) sentezlenmiş, ultraviyole ışık ve fotobaşlatıcı kullanılarak mikroakışkan çip üzerinde bir üç boyutlu hidrojel yapı oluşturulmuştur. Bu tez çalışmasında kullanılan strateji ile, mikroakışkan çip üzerinde hidrojel üreterek yapılan ko-kültür çalışmalarına kolay ve başarılı bir alternatif sunulmuştur.

Sentezlenen GelMA'nın kimyasal içeriği fouirer transform infrared spektrofotometre (FTIR) ile belirlenmiş, yüzey morfolojisi taramalı elektron mikroskobu (FESEM) ile incelenmiş, yapısal özellikleri ise atomik kuvvet mikroskobu (AFM) kullanılarak analiz edilmiştir. Üretilen GelMA hidrojel yapısında nörovasküler bir ko-kültür oluşturulmuş, çip içerisindeki hidrojeldeki hücre canlılığı ve çip dışındaki hidrojelde hücre canlılığı ile karşılaştırılmıştır. Çip içerisindeki hidrojelin emme kapasitesi görsel olarak incelenmiş ve hidrojelin porlu yapısı 72 saat boyunca hücre hareketleri immunoflörasan mikroskopu ile izlenmiştir.

Bu çalışmalar sonucunda sentezlenen GelMA'nın istenilen biyomekanik özelliklere sahip olduğu ve yedi güne kadar 80% üzeri hücre canlılığı sağladığı gözlemlenmiştir. Ayrıca GelMA fotopolimerleşme kullanılarak mikro akışkan çipte üretildiği takdirde de üç boyutlu hücre kültürü çalışmaları için geçerli bir alternatif platform olma özelliğini korumakta ve yapısal bütünlüğünü uzun süre korumaktadır.

1. INTRODUCTION

Biomaterials and biomedical devices are being produced for healthcare applications for years. Design logic of those devices are based on healing or replacing a tissue, organ or a cellular function. Those devices can be designed to carry bioactive items such as drugs, growth factors (GFs) and biomolecules. However, those devices, before the development of tissue engineering methods, were unable to carry biological entities, such as cells. With the invention of tissue engineering methods, biodegradable devices that are capable of carrying cells to a tissue became very appealing. Tissue Engineering is an interdisciplinary field that combines natural science with engineering in order to restore, improve, maintain or replace tissue functions. For this purpose, those devices are designed to blend with the tissue when implanted and able to leave no trace on the tissue after a certain time. Those tissue engineering strategies and devices became favorable compared to conventional implantation methods, as it is seen more promising for long term. Three essential components for tissue engineering are scaffolds, cells and bioactive molecules. Basic purpose of a scaffold is to host seeded cells, becoming a mechanically and biologically viable microenvironment for tissue engineering applications. Those scaffolds have porous design which allows penetration of cells, nutrients and growth factors. Also, their surface properties should be chemically valid, being adhesive enough for cell attachment. The second component of tissue engineering applications, cells which seek a surface to attach and grow. This is also referred as anchor-dependency and most of animal cell lines require to attach a surface to grow. Those cells can be either primary cells or stem cells. Primary cells are the cells directly taken from a tissue and didn't undergo any subculturing process. Those cells are mature, differentiated cells which are divided in two classes; autologous and allogenic cells. Autologous cells are the patient's own tissue cells which are taken from the healthy part of a tissue. Those cells show no pathogen transmission or no chance of rejection by body as it is directly taken from the patient. But the drawback of this method is pain and the limited availability. Allogenic cells remove those drawbacks as they are taken from another

human donor. But the drawback of this method is chance to carry pathogen transmission and high chance of rejection from other tissue. Different from autologous and allogenic cells, stem cells are the cells that are capable of growing into various types of tissues or even organs. Being able to divide limitlessly and being able to form new stem cells or differentiating to another type of cell makes them ideal tools for cellular repair and regeneration. Bone marrow is an essential source to derive stem cells. Lastly, the growth factor should exist on a scaffold structure to regulate cellular activities. GFs stimulate growth, and they are also responsible for cell division and cell survival. Those protein molecules are essential to engineer and grow a tissue model. Only after selecting the ideal properties for three essential components; type of scaffold, cells and growth factors, tissue engineering can be done. Those three components have various combinations that result in different outcomes, which are being used at different tissue engineering applications. Recent advancements in biomaterial science and nanotechnology allow scientists to engineer new and more precise tissues with using more advanced tools and methods.

1.1. Tissue Engineering Scaffolds

Tissue Engineering (TE) is an interdisciplinary field that combines natural science with engineering in order to restore, improve, maintain or replace a tissue functions by developing biological scaffolds. Those scaffolds fill the tissue void and provide biomechanical support to promote formation of the tissue. They work as cell carriers that create an environment that promotes cell growth while providing optimal structural support to engineer a tissue. As cell surface receptors transduce signals into cells from the extracellular matrix (ECM), they also regulate cellular functions such as survival, growth, migration, and differentiation, mimicking the cellular environment [27]. To build an optimal scaffold, one of the main strategies is engineering biomimetic structures, such as hydrogels, as they have the ability to intimidate the native ECM successfully. Those structures are commonly being used in tissue engineering and drug delivery applications.

The scaffold can be made with using various materials with different forms. Types of scaffolds are divided in two, natural and synthetic. Synthetic scaffolds are made with

using polymers such as polyamides, polyesters and polylactides. Natural scaffolds are made with using biological polymers, like collagen, gelatin, chitosan and cellulose.

There are various strategies to create scaffolds for in vitro tissue engineering to mimic ECM, such as electrospinning, 3D printing and various molding techniques [22; 47; 55]. The design of those scaffolds plays vital role on building the optimum conditions for cell nutrition, growth, medical stability and transportation of nutrients. Besides those, hybrid (cell-scaffold) constructs are being used to encapsulate cells with using crosslinking techniques [21].

1.2. Design requirements of Scaffolds

Design of the scaffold is a vital part of tissue engineering and regeneration. There are many considerations and parameters to design an optimal scaffold that promotes cell growth, nutrition and attachment. Besides those properties, an ideal scaffold should be able to meet several requirements as listed below [38; 60; 61]:

1. *Biocompatibility*, as a primary requirement for any type of a scaffold. As cells going to be seeded on a scaffold, the scaffold should give an appropriate bio-response.
2. *Biodegradability*, as native tissues are going to replace the scaffold that needs to be removed from the site.
3. *Optimum degradation speed*, as degradation rate should be proportional to the regrowth of a new tissue.
4. *Ideal mechanical properties*, as mechanical properties of a scaffold seeded with cells should match the host tissue as close as possible.
5. *Having optimum porosity*, as porous structure of a scaffold is vital for efficient delivery of nutrients and growth factors to make the cells grow.
6. *Ideal surface properties*, as surface of a scaffold should promote cell attachment, growth, proliferation and ECM formation. In order to do that, surface modification strategies can be considered [32].

7. *Ease of production*, as easily repeatable processes with simple design strategies are preferred to produce scaffolds in short time with minimal chance of production errors.

1.3. Extracellular Matrix (ECM)

Extracellular matrix (ECM) is responsible for structural stability of tissues and regulating biological events such as cell-matrix and cell-cell interactions. ECM plays crucial role on various cellular activities such as migration, survival, proliferation, morphogenesis, angiogenesis [41]. ECM consists of fibrillar proteins (including collagens), glycoproteins, proteoglycans, laminin, elastin and fibronectin.

Collagen is the most common type of fibrous protein in mammal body. Mass of collagen is around 30% of total protein mass in mammals [12] . Collagen is responsible for mechanical stability, adhesion and development of tissues. *Elastin* gives elasticity property to tissues under mechanical forces that causes stretch movement [74]. For example; lung and vascular vessels are subjected to that repeating stretch in regular frequencies. *Fibronectin* (FN) is a multidomain macromolecule that is responsible for cell attachment and migration as it consists binding sites. FN can be stretched several times, increasing its length. With this stretching, it causes pleiotropic changes in cells as it exposes more integrin binding sites [64]. *Proteoglycans* are responsible for stabilizing ligand-receptor interactions, regulating cell proliferation, growth factor, endocytosis and signal regulation [5]. They also protect growth factors from proteases and control the cell migration. *Laminins* are structural proteins, playing critical role on cell attachment and differentiation, tissue survival, cell movement and shape, also they are responsible for organizing interactions of basement membranes.

1.4. Hydrogels

Hydrogels are three dimensional crosslinked polymeric structures with hydrophilic properties. They are half solid-half liquid like structures with capacity to swell huge amounts of water while being able to protect their shapes [51]. They are one of the most important biomaterials as they have great biocompatibility due their large water

content, causing minimal inflammatory responses and tissue damage [48]. They can absorb and retain large quantities of aqueous fluids. Also, they have ability to change their chemical structures depending on physical stimuli applied to them such as pH, temperature, salt concentration change etc. [62] .Their structure mimics the permeability of the ECM for optimal transport of oxygen, nutrients and waste products [46]. As hydrogels have cellular biocompatibility, they have variety of use as cell scaffolds [39] or drug delivery vehicles [63].

Liquid composition and inner structure of a hydrogel influences the behavior of itself. Those behaviors include swelling efficiency of a hydrogel. For example, crosslinking density and increased amount of porosity increases the swelling rate of a hydrogel, whereas ionic polymers with monovalent ions or hydrophilic crosslinkers increase swelling capacity [50].

Hydrogels can be synthesized via crosslinking of polymers using physical stimulus. Those triggering forces include photopolymerization, radiation, heat reactions and chemical reactions. Those major gelation processes have pros and cons. Triggering the gelation process with using pH change cause toxicity and inability to control the crosslinking density, which makes harder to adjust the hydrogel's properties [6]. Also, heat treatments should be made with temperature of human body temperature for ideal biocompatibility. Photopolymerization, done with ideal parameters (duration, light source, polymer solution) is considered optimal compared to other results. It also allows easier control over crosslinking density with minimal toxicity.

1.5. Photopolymerization

Hydrogels can be manufactured by crosslinking of polymers. Photopolymerization using radiation, chemical reactions, applying heat are some of crosslinking methods used to synthesize hydrogels [59]. Photopolymerization process is usually done by using a photoinitiator (PI), irradiation with using the optimum wavelength for cell survivability and optimal gel structure as free radicals dissociate from PI during irradiation [19]. Their radicals create covalent bonds that crosslink the hydrogel network usually between seconds and a minute [31].

A photopolymerizable system is made of a monomer and two photopolymerization initiating tools; a PI and a light source. Monomers are mostly organic molecules that interact with other molecules to form polymers. Whilst bifunctional monomers can only form linear, chainlike molecules, polyfunctional monomers have capacity to form chemical bonds with multiple monomer molecules. PIs are being used in photopolymerization process. PIs allow the use of light to polymerize and cure the materials, or to form crosslinked hydrogels. Visible, infrared or UV light can interact with PIs and create free radicals which initiates polymerization process. The first synthetic light sensitive polymers were developed at Kodak [43] and currently it is being used for various applications, such as coatings, printing materials, sealants, microelectronic resists and dental applications [67].

Photopolymerization has faster curing rates compared to conventional polymerization techniques at room temperature. Using light instead of using intense heat makes them preferable over those conventional techniques. Other advantages include very high reaction rates, spatial control of polymerization, low energy input and chemical versality [14].

Fabrication of polymers *in situ* is advantageous as it is easier to form complex shapes that contains tissue structures, this property makes polymers widely used in biomedical applications. But a major drawback of polymerization is the difficulty to adapt biologically optimal conditions while forming the polymers. Biological structures require specific properties in narrow conditions, such as temperature, pH and minimal toxicity. But as photopolymerization conditions is milder than conventional polymerization techniques, those drawbacks are non-existent in most of the polymerization applications [37].

1.5.1. Light sources

Various types of light sources are being used to produce polymer networks. Ultraviolet (UV) light sources, light emitting diode (LED) lamps, halogen lamps and different types of laser lamps are being used for photopolymerization. Those lamps have different emission wavelengths therefore have different effects on photopolymerization. For tissue engineering, UV and halogen lamps are the most

common light sources among all as they have optimal properties for drug delivery and cell encapsulation applications [16; 24; 73]. Other light sources such as plasma arc and LED lamps are being used at dental applications [3; 36], whilst laser lights are being used at surface patterning or micro/nanopatterning applications in general [58; 71].

1.5.2. Photoinitiators (PI)

Photoinitiators (PI) are light sensitive initiators of photopolymerization process triggered by a light source. They produce reactive species with absorption of the light. There are various types of PIs that are being used for different applications, and there are few of them used for tissue engineering applications and cell encapsulation purposes [37].

PIs are divided in two groups, Type I and Type II respectively;

Type I PIs release two free radicals upon light exposure. Those generated radicals trigger the polymerization as they are produced through a unimolecular bond cleavage. Photolysis happens at the most fragile spots in their chemical structures, mostly at CO-C bond of aromatic carbonyl compounds [25]. Type II PIs are less efficient overall compared to Type I PIs as they experience a biomolecular reaction. To produce free radicals, triggered PIs abstract hydrogen from donor molecules which initiates this biomolecular reaction.

There are various types of PIs. Irgacure 2959, VA-086, LAP and Eosin Y are the most commonly used PIs as they have high crosslinking abilities [44; 49]. Their solubility, curing time and ideal concentration for cell encapsulation vary among each other. Also, the ratio of PI in the pre polymer solution got vital importance as increased amount of PI in the solution tends to reduce the cell viability as seen at (Figure 1.1).

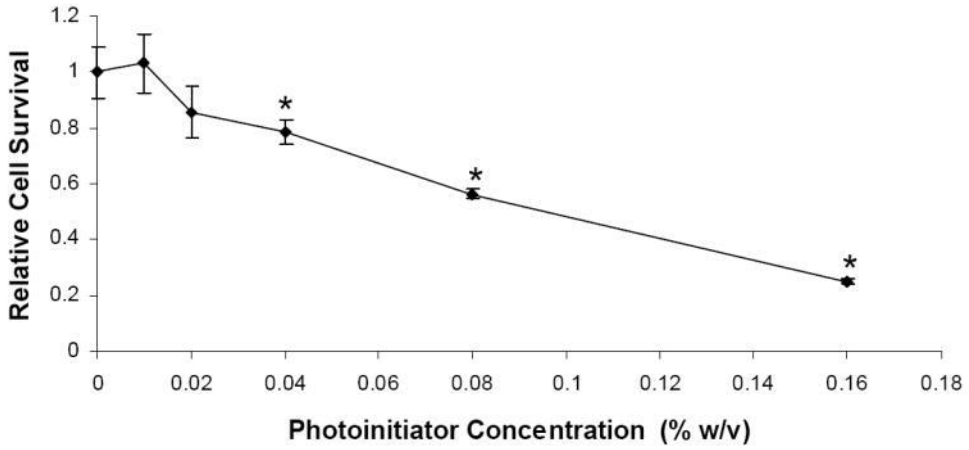


Figure 1.1 : Relative cell survival values of human aortic smooth muscle cells based on PI (Irgacure 2959) concentration in absence of UV light [40]

When considering cell viability or building a cell encapsulation system those parameters should be considered.

1.5.3. Photopolymerization of hydrogels

Two common methods are being used to manufacture hydrogels with using photopolymerization techniques. One of them is bulk photopolymerization. Bulk photopolymerization process is based on using a PI and dissolving it in a hydrogel solution. Afterwards the light source, such as UV is being applied under specific conditions (time, intensity, range). This makes the hydrogel precursor solution to turn into hydrogels. Second method is called interfacial polymerization which is used to create hydrogel linings on tissues. This technique involves adsorption of PI onto the surface of the tissue/cells. The hydrogel precursor is used marker on the surface at this method as when it is exposed to the correct wavelength of light, the polymerization occurs [37]. This method shows better structural/biological efficiency compared to bulk photopolymerization method as nutrient transport becomes more efficient [45].

Besides choosing a method for photopolymerization, pre-polymer solution also should have desired parameters for optimal cell encapsulation and hydrogel formation. Cell viability and gel quality in cell encapsulation and hydrogel formation have vital importance. There are several parameters such as pH and solvent as it directly effects toxicity of the environment. The solvent should be prepared with non-toxic solutions

and pH level should be same with blood as the environment should mimic the body. Under ideal pH conditions, blood can transport nutrients without causing any toxicity. Besides biocompatibility parameters, mechanical priorities of pre polymer solution is crucial to produce ideal structures. The solution of polymer concentration affects the rigidity of the gel structure. Stiffness of the gel should be optimal as too much stiffness might prohibit cell migration in the gel or too less stiffness can cause weaker structure which loses its' gel structure easily [40]. Besides biomechanical properties, solubility of the PI should be considered too. High temperature heating and using vortex would help PI dissolve in water quicker and easier.

1.5.4. Ultraviolet (UV) curing parameters

In this study, Irgacure 2959 is selected as a PI, as it provides optimal cell viability and minimal cytotoxicity for UV photopolymerization process [13]. To initiate photopolymerization, UV was used as the light source, as better cell viability results was reported compared to other light sources [57]. Power of the curing lamp in this study is 200 W and UV light is applied was applied from top part of the microfluidic chip as distance from top layer to channels is shorter than the distance from bottom layer to channels, which increases the efficiency of the UV slightly. The intensity of the UV can be adjusted between 30W/cm^2 to 2MW/cm^2.

1.6. Gelatin

Collagen is the most abundant protein for mammals, and it has many different functions at different parts of body. The most abundant types of collagens in human body are type I, type II and type III collagens. Structural shape of type I collagen is based on three spiral chains and those chains have around 1.5 nm width and 300 nm length [65]. This three-spiral polypeptide chain structure causes low antigenicity which limits its application in bioengineering. Besides that, hydrophobicity properties of the building amino acids, collagen become insoluble as it is very hydrophobic. Even if it gets solubilized, collagen loses its mechanical properties such as strength and stiffness.

Gelatin is a protein derived from part I collagen. They are polypeptide with biodegradable properties derived from hydrolysis from natural polymer collagens [65]. Because of the hydrolysis process, gelatin got higher solubility and it is solvable in water. Gelatin based hydrogels show excellent biocompatibilities, ability to degrade rapidly and ability to mimic ECM accurately. Therefore, they can mimic ECMs and have the role of maintaining cellular structural stability and the integrity of tissue networks such as blood vessels or bones.

A drawback of gelatin is their instability *in vivo*. Their melting temperature is lower than body temperature so to stabilize macromolecular structure and thermomechanical properties crosslinking is applied on gelatin. This improves structural stability and degradability on the host tissue [42].

1.7. Gelatin Methacrylate (GelMA)

Gelatin methacryloyl (GelMA) , firstly defined by Van Den Bulcke et al [1], is one of the most promising hydrogels for 3D cell culture researches due its high biocompatibility, low cost and ability to degrade. GelMA is derived by deriving the gelatin with using methacrylic anhydride, modifying lysine and hydroxyl residues with methacrylamide. The main gelatin molecule keeps protecting its stability as biomaterial after the derivatization process. Thus, GelMA as a hydrogel can provide a biocompatible environment that promotes cell growth, adhesion and proliferation.

The modification of methacryloyl allows GelMA to polymerize with UV light, using PI. This creates a methacryoyl backbone with covalent crosslinking, causing thermal and mechanical stability and negates the major drawbacks of conventional gelatins [34]. Also, it’s possible to tune mechanical properties of GelMA, increasing surface adhesion or tuning the pore sizes, which are crucial for cell adhesion and viability [2]. (Figure 1.2) explains photopolymerization process done with using a microfluidic chip.

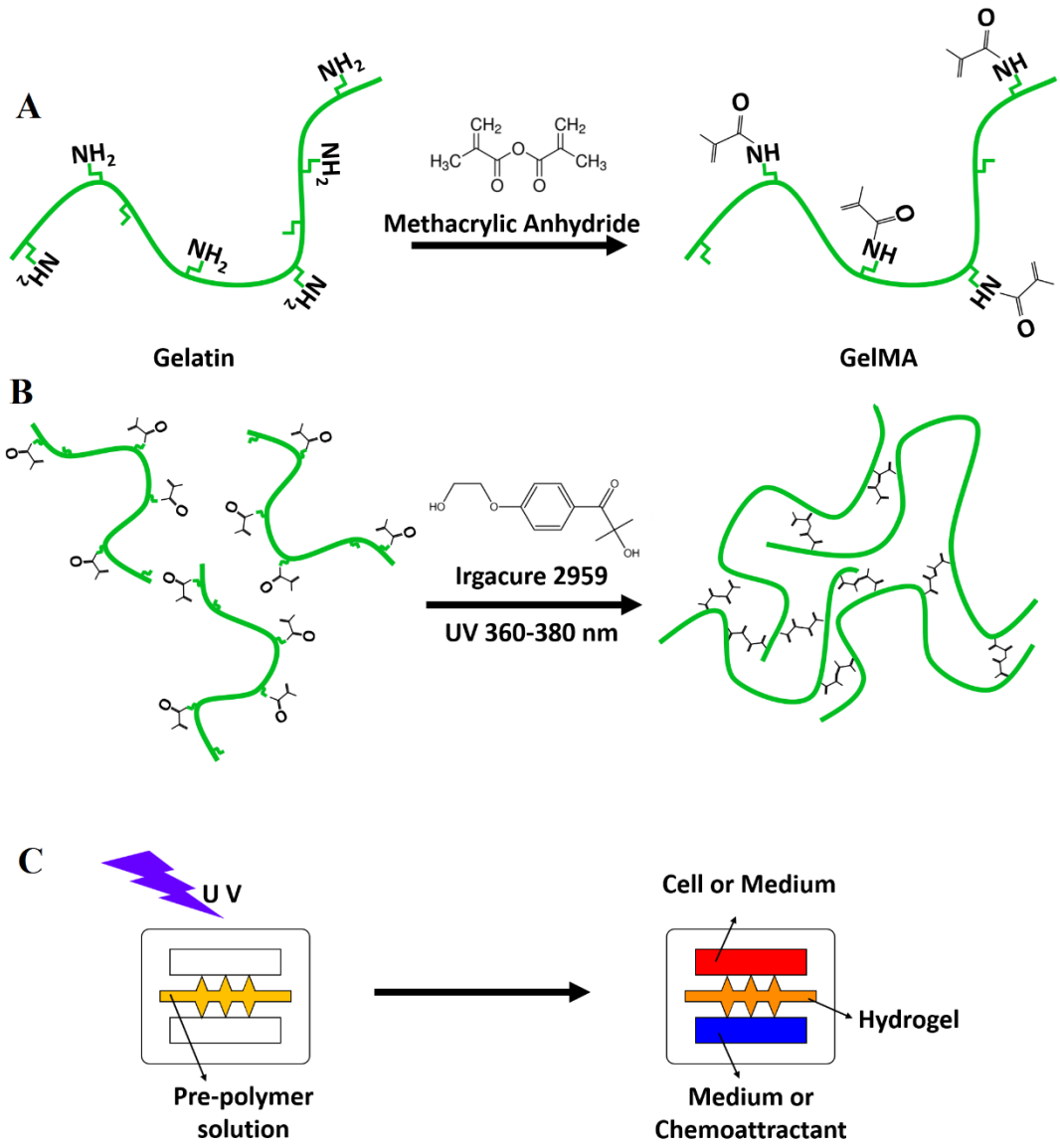


Figure 1.2: Synthesis and fabrication of a photocrosslinked GelMA based hydrogel inside of a microfluidic device. (A) Methacrylic anhydride (MA) was reacted with gelatin. This process was made to add methacryoyl on amine and hydroxyl groups. (B) Photocrosslinking reaction using Irgacure 2959 as a photoinitiator. The free radicals generated by the photoinitiator initiated the photopolymerization. (C) Schematic of formation hydrogel inside of the middle channel of a microfluidic device.

GelMA can also be manufactured on different architectures. Those different architectures can be created via using different patterned mechanical surfaces used as molds. Besides those, with using different photomasks it is possible to create variety of patterns. Bioprinting and using microfluidics also lets the user to pattern those

architectures on microscale. Versatility of GelMA includes creating hybrid hydrogels too. It is possible to mix GelMA with various nanoparticles or other polymers. By doing that, it is possible to create biological networks with ideal biological properties for bio applications [29]. Those properties allow GelMA to be used not only at tissue engineering, it makes GelMA also useful at biosensor field, drug delivery applications, cell signaling applications [34].

1.8. Advantages of 3D Cell Culture over 2D Cell culture

Earliest cell culture models were developed by Harrison in 1907 [56]. Since his experiment on nerve fibers, cell cultures are being used to experiment growth and differentiation of cells. Cell culture researches are vital part of cell biology field as it makes researchers understand disease mechanisms, drug effects and efficiencies, dose controls, tissue morphology and other properties of tissues.

For years, 2D cell cultures were used to experiment those properties of cells or tissues. This protocol is based on seeding cells over a flat surface and growing them under appropriate conditions. Even 2D cell cultures have advantages like simplicity and low cost, they are unable to mimic a 3D environment efficiently. For tissue engineering, 2D cell Culture strategies are unable to show cell-cell and cell-ECM interactions which are responsible for various cellular actions such as; proliferation, differentiation, protein and gene expressions, responses to stimuli, etc. [11]. While cells are isolated from tissue and added to 2D conditions, morphology of cells get altered, cell division mode and diversity of the phenotypes gets reduced [52]. As morphology changes, cellular activities, organization and functions changes, making cell response and behavior far from their activity in their natural environment.

Unlike 2D cell cultures, newly developed 3D cell culture strategies develop more realistic environments for tissue engineering studies. Since Hamburg and Salmon's first 3D cell culture experiment in the 1970s [26], this technology is being widely used and developed as it gets cheaper and more efficient with advances in bioengineering. Some advantages of 3D cell culture methods are more accurate morphology, higher viability, better proliferation and better differentiation.

In 3D cell cultures, cells are ellipsoids, unlike cells in 2D culture which are flat. Because of that, in 3D cultures, morphology, integrity and alignment of cells are more accurate as cells grown in monolayers doesn't have an altered morphology [10]. As cell-cell interactions in 3D Cultures are more accurate and prominent, viability is higher and cells are healthier overall compared to 2D environment [17]. Better cell functions are observed with using the appropriate scaffold based 3D cultures [9]. As shear effect is lower at matrix-based culture models, growth rates are observed higher and apoptosis rate is observed lower in various type of cells, such as human umbilical vein endothelial cells (HUVEC) [18]. But discussions are still ongoing on proliferation as some cell types like airway smooth muscle cells show lower proliferation because of morphological differences [15]. (Figure 1.3) shows a brief visual comparison of cell growth at 2D and 3D environments.

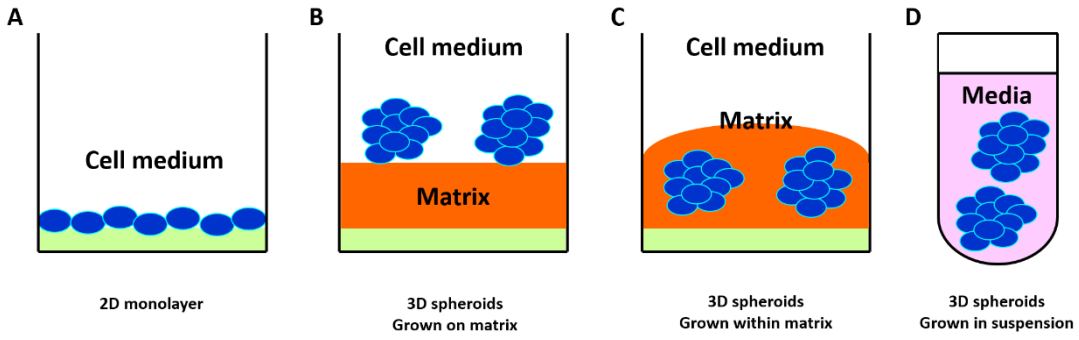


Figure 1.3: Schematic diagrams of the traditional two-dimensional (2D) monolayer cell culture (A) and three typical three-dimensional (3D) cell culture systems: cell spheroids/aggregates grown on the matrix (B), cells embedded within the matrix (C), or scaffold-free cell spheroids in suspension (D).

1.8.1. Co-culture systems

Cell co-culture systems have various applications for studying cell biology, tissue - tissue interactions, cell communications between various group of cells. Co-cultures are commonly used for drug and dosage experiments being tested on tissues as it is possible to make a realistic environment with tissue models with the ability to examine cellular interactions [69]. For example; it is possible to establish complex models such as blood-brain barrier with mimicking neurovascular units [4]. Just like conventional mono-culture models, ECM is the key part of building natural-like systems.

1.9. Microfluidic Devices For Cell Culture Applications

Microfluidic devices allow better control over fluid flow and mechanical effects on nanoscale compared to conventional cell culture techniques. They allow users to spatially control and pattern co-cultures, with using hydrogels [68] or adhesion molecules [20]. Besides those features, microfluidics provides faster analysis, using less ingredients/fluids and ability to do real-time imaging. Also both adherent and non-adherent cells can be cultured in microfluidic platforms [28; 53].

The aim of microfluidic devices is to create more accurate systems that is closer to *in vivo* structures with ease of use and manipulate. Controlling the microenvironment is one of those features and microfluidic devices allow users to alter and manipulate the microenvironment easier by controlling physical effects such as flow rates, cell gradients as well as ability to model biomechanical activities of biological events such as capillary network formation described at Figure 1.4.

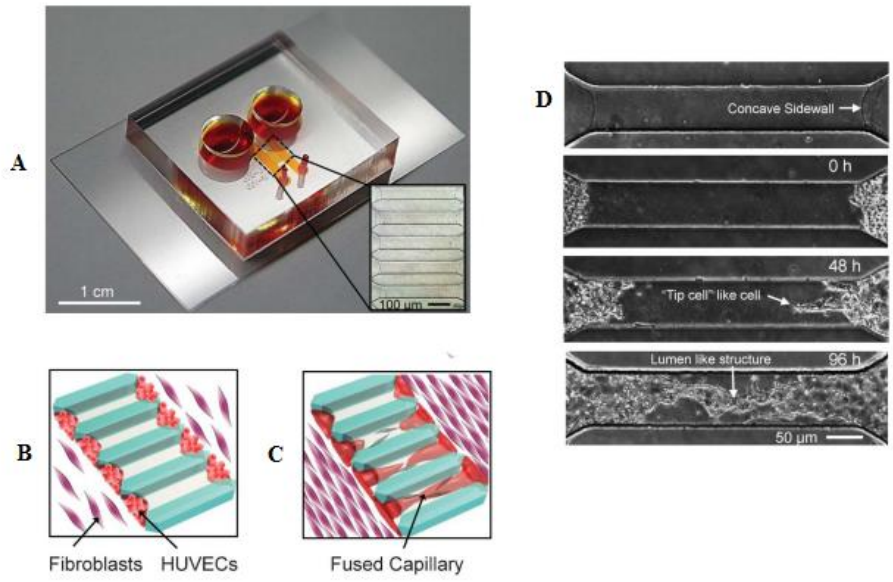


Figure 1.4: Capillary formation inside a microfluidic chip. A) Microfluidic device designed to form capillary networks. B) Schematic representation of inner microstructures. Human endothelial vein cells (HUVEC) were loaded on side walls and Fibroblasts were loaded from inlet channels. C) HUVEC were grown from sidewalls and attach to each other. D) Image of forming capillary networks [70]

Combining hydrogels with microfluidic devices offers more realistic, *in vivo* like environments in microfluidic chips. The 3D matrix structure in microfluidic channels are patternable with using microstructures like pillars, ridges in desired patterns [35]. This makes hydrogel structures spatially controllable, even making possible to create lumens inside of chips [7]. Depending on the pattern of the channels, it is possible to create hydrogel blocks inside of channels to co-culture or tri-culture with desired patterns.

Vasculature models are commonly used for the cell culturing applications in microfluidic devices. Those models are based on using endothelial cells and growing them up inside of a hydrogel or against the hydrogel. Also, metastatic cascades which are related with vascular system can be modeled on microfluidic chips with culturing cancer cells with endothelial vessels. Events like migration [33] , intravasation [30], extravasation [35] and angiogenesis [66] are observable on those models.

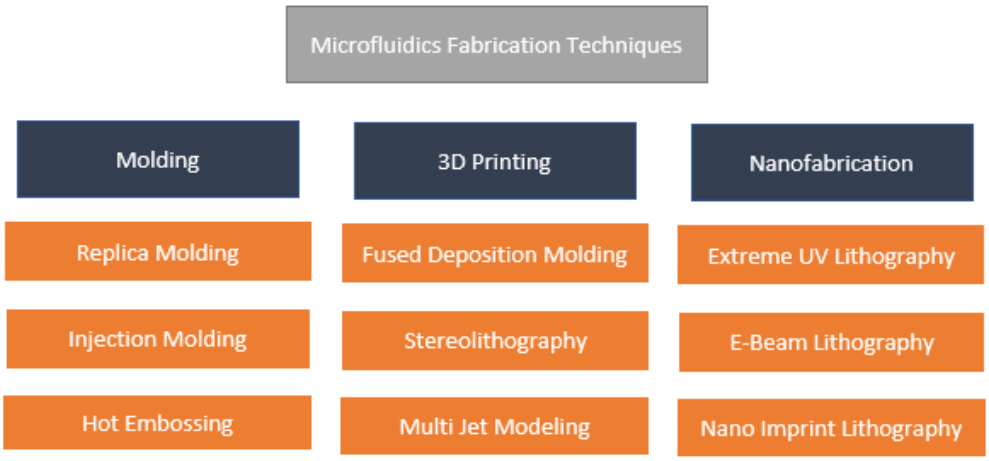
Polydimethylsiloxane (PDMS) is the most common material of microfluidics for cell culture applications. It is transparent, which makes the observation of channels with

microscopy easier, it has low autofluorescence and it is deformable as it has lower young's modulus compared to its alternatives such as glass which makes it preferable when observing very low forces and mechanic interactions of cells. It is easy to mold and produce as well as it is gas permeable which is crucial for cell culture applications.

1.9.1. Manufacturing methods for microfluidic chips

There are many methods to manufacture microfluidic devices using PDMS. Those methods can be divided into three branches; molding, 3D printing and other nanofabrication methods as seen at (Table 1.1).

Table 1.1: Commonly used microfluidics manufacturing methods.



Molding techniques can be divided into three categories, replica molding, injection molding and hot embossing. Replica molding is based on using photolithography to create a silicon mold and pouring PDMS on top of the mold. It is also called soft lithography. Injection molding has same working principle on bigger scale injection molding techniques. The mold is designed with two parts, aligned to each other with empty space in between which is going to be filled with PDMS through the input space that allows fluid to get inside of the mold. After this process, mold gets cooled down and separated. This method is ideal for fast manufacturing. Hot embossing is based on

using intensive heat and contact pressure. Just like stamping, a stamp filled with PDMS is transferred on a surface.

3D Printing strategies are getting more popular as the 3D printing technology is rapidly advancing. Increased layer resolutions, faster printing durations, newer methods make 3D printing more desirable on nano/microfabrication and microfluidic technology is easily producible with 3D printing. Fused Deposition Molding is the oldest 3D printing method to produce microfluidic chips. In this method, the material is melted into the nozzle and extruded layer by layer as it binds before cooling. Stereolithography is based on photopolymerizing a resin with using a light source. The mold or the structure itself, made with a Computer Aided Design (CAD) software is being used as the pattern of the light source that will polymerize the resin, making it solid. Multi Jet Modeling also uses a photo sensitive resin and this resin is released from inkjet printhead droplet by droplet. Then it's cured by a light source inside of the printhead which lets user to manufacture high resolution micropatterns [72].

Nanofabrication methods are being used in various nanomanufacturing strategies. Those methods include microfluidic devices too. Extreme UV lithography (EUV) is a technique that is similar to standard lithography processes, the difference is in EUV, wavelengths are way shorter than lithography processes as standard wavelength is 13nm [8]. To create light at this size, laser is being used to create a plasma and this plasma emits EUV light. This light is then reflected from a mirror to a system of reducing mirrors. Then this light is focused on a photoresist, and etching begins afterwards. Another method is Electron Beam Lithography (EBL). It is a method based on using scanning electron microscope (SEM). Different from other lithographic processes instead of light, electron beam is used to expose the resist. This electron beam increases the solubility of the resist, creating the pattern. Lastly, nanoimprint lithography (NIL) is being used as a cheap manufacturing method. NIL is based on using a mold, which is pressed into a resist. After deforming the resist material by hardening the stamp with using physical or chemical procedures, the mold is being removed from the resist and designed pattern remains.

1.9.2. Parameters for co-culturing cells in microfluidic platforms

In addition to parameters conventional culturing methods, culturing in microfluidic devices got a few more parameters, mostly related with mechanical properties of the microfluidic device. Conventional culturing parameters can be listed as:

- Selection of the cells
- Selection of the appropriate medium
- Selection of the seeding method
- Anchorage dependency
- In vitro cell senescence
- Selection/design of the appropriate microfluidic chip

In co-culturing, primary culture or cell line should be selected first. According to cell types, basal media for the optimal growth must be selected. Basal media contains a balanced salt solution which helps buffering the medium at physiological pH, maintaining membrane potential, maintaining osmotic pressure and help cell attachment. Also, it contains a buffering system, nutrients such as amino acids, carbohydrates, vitamins and also antibiotics to avoid contamination. Anchorage dependency is important as some cell types can be independent (anchorage-free), which grows without attaching to a surface as anchorage dependent cells require additional processes such as trypsinization to become independent from the surface that they attached. In vitro cell senescence (cell aging) is also crucial as cell characteristics may alter depending on age of a cell type as seen at Table 1.2, which can be determined by amount of passages (subculture).

Table 1.2: Changes in characteristics of fibroblasts with increasing amount of passaging.

• Increased cell doubling time
• Decreased adhesion to substrate
• Altered cytoskeletal organization
• Descreased efficiency of protein synthesis
• Descreased amino acid transport
• Insensivity to growth factors
• Shorter telomeres

When co-culturing cells in microfluidic devices, instead of selecting appropriate platforms for conventional methods such as selecting ideal petri dishes or well plates, appropriate chip design should be selected according to purpose. For example, microfluidic devices with appropriate amounts of channels and contact points should be selected for the experiment. Also, devices with micromechanical tools, such as micropumps or mixing chambers can be designed depending on the purpose of the experiment.

2. MATERIALS AND METHODS

2.1. Devices

2.1.1. Invasion Chemotaxis Chip (Initio Biomedical, Turkey)

Invasion Chemotaxis Chip (IC-Chip) is a microfluidic chip, made of polydimethylsiloxane (PDMS). This gas-permeable chip consists of three channels, two of them (bottom and top) being same size with same volume (12 µl approx.) and a middle channel which got three intersection points with each side channel. This middle channel got approx. 10 µl volume and is an ideal environment to test hydrogels as it is connected to both side channels. As the microchip is made of PDMS, it is biocompatible and it's transparent with low autofluorescence which makes the chip ideal to use for fluorescence microscopy as seen at (Figure 2.1).

Figure 2.1: Invasion Chemotaxis Chip (IC-Chip)

2.1.2. Omnicure S2000 Spot UV curing system (Excelitas Technologies Corp., USA)

Omnicure S2000 Spot UV Curing System consists a 200 Watt UV lamp and a probe. This UV curing lamp can reach outputs up to 30 W/cm^2. It has a standard UV curing system filter of 320-500 nm and it can be configured to various values ranging between 250-500 nms. In this research, this device, seen at (Figure 2.2) was used to initiate the

polymerization process to create the hydrogel that got ability to encapsulate the cells inside of a 3D hydrogel structure.

Figure 2.2: Omnicure S2000 Spot UV Curing Device

2.1.3. Class II microbiological safety cabinet

Microbiological Safety Cabinet (MSC) is a device used to avoid hazardous particles and aerosols generated by materials used in microbiological experiments. It is also used to prevent contamination and to provide a clean work environment for cell culture applications. Class II cabinets protects both work and the operator and they are ideal type of MSCs for cell culture applications.

2.1.4. CO_2 incubator (Memmert GmbH, Germany)

The CO_2 Incubator maintains the three critical parameters to provide optimal cell growth conditions. Those parameters are temperature, humidity and ratio of carbon dioxide. For mammalian cell culture, usual temperature is 37 °C, the humidity level is 95%, and the CO_2 ratio is 5%.

2.1.5. Centrifuge

The centrifugal force of a centrifuge is used to separate liquid and/or solid substances based on their different densities by pushing the heavier materials towards outside of the vessel. In this research centrifuge is used for pelleting cells, purifying them from the medium or the immunofluorescence dye solution.

2.1.6. Multiskan FC microplate photometer (Thermo Fisher Scientific, USA)

The microplate reader is used to measure absorbance values for cell viability applications. To measure the cell viability, sample is placed into a microplate after using a cell viability protocol of an assay such as alamarBlue and afterwards values such as absorbance, fluorescence intensity and luminescence values are measured.

2.1.7. Müve NB9 water bath (Müve, Turkey)

Water bath systems are used to thaw or warm the experiment reagents such as culture mediums, serums or related liquids prior to use. Even though heat dispersal isn't as effective as heat dispersal in an incubator, water bath got less risk of contamination from water. In this experiment, the water bath is operated at 37°C.

2.1.8. Zeiss GeminiSEM 500 (Carl Zeiss AG, Germany)

Zeiss GeminiSEM 500, seen at (Figure 2.3) is a Field Emission Scanning Electron Microscope (FESEM) used to image surface morphology and measure pore sizes in high resolution.

Figure 2.3: FESEM imaging setup

2.1.9. Olympus CX22 microscope (Olympus Life Science, USA)

In this research Olympus CX22 Microscope, shown at (Figure 2.4) was used to observe cell attachment and growth before and after incubation.

Figure 2.4: Olympus CX22 Microscope

2.1.10. Olympus BX51 fluorescence microscope (Olympus Life Science, USA)

Olympus BX51 Fluorescence Microscope was used for immunofluorescence imaging for cell localization and cell viability studies in this research. (Figure 2.5) shows BX51 fluorescence microscope sending a specific wavelength during imaging process.

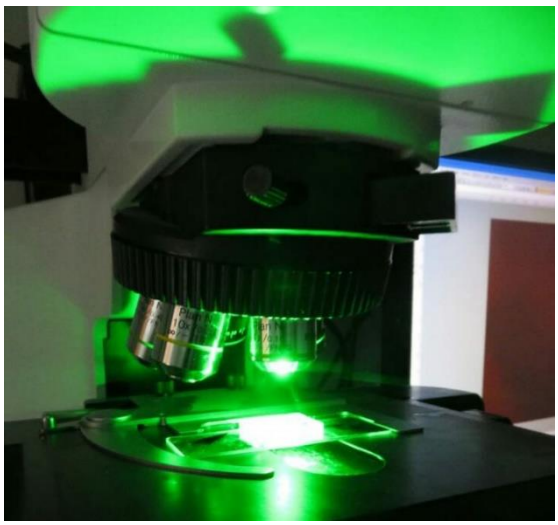


Figure 2.5: Olympus BX51 Fluorescence Microscope

2.2. Methods

Seven main steps were followed in this experiment.

1. GelMA Synthesis

2. Characterization of lyophilized and hydrogel GelMA
3. Cell encapsulation inside of hydrogels.
4. Viability tests of encapsulated cells in 3D matrix.
5. Immunostaining of neuroblastoma and endothelial cells.
6. Cell encapsulation and building the 3D hydrogel structure inside of the chip.
7. Loading endothelial cells inside of channels.
8. Fluorescence imaging of the cells.
9. Viability tests inside of the chip.

2.2.1. GelMA synthesis

Gelatin was added to the Dulbecco's phosphate buffer saline (DPBS) solution (Biological Industries, USA) of 100ml with 10% (w/v) concentration. Afterwards, the solution was dissolved at 60°C using a magnetic stirrer. After the solution was dissolved, methacrylic anhydride with 8% (w/v) concentration was added with a rate of 1ml/min. The solution was kept stirring in 50°C for 3 hours. The solution got diluted five times more, adding more DPBS afterwards to stop the reaction. As the reaction caused salts and other unwanted compounds to appear, a dialysis procedure was held for 7 days. After the dialysis, the solution was lyophilized and stored in -80°C with no light contact.

2.2.2. Field emission scanning electron microscopy imaging of GelMA

Field Emission Scanning Electron Microscopy (FESEM) was used to observe the surface morphology of the GelMA. Lyophilized GelMA was coated with palladium nanoparticles and its surface morphology was observed with Zeiss Sigma 500 VP FESEM at Izmir Biomedicine and Genome Center (IBG), Turkey.

2.2.3. Surface coating of coverslips

To increase the surface adhesion of the glass and to give more structural stability to gel, surface of the coverslips was coated with 3-(Trimethoxysilyl)propyl methacrylate (TMSPMA) (Sigma Aldrich, China). 10% sodium hydroxide (Merck, Germany) solution were applied coverslips for 1 hour. After getting washed with distilled water,

nitrogen gas applied to dry the coverslips. Then the coverslips were dipped into TMSPMA in 80°C for 12 hours. The coverslips were cleaned with ethanol afterwards and dried again using nitrogen gas. The treated coverslips were used in 15 days after getting coated.

2.2.4. Preparation of the pre polymer solution

To initiate the photocrosslinking process, photoinitiator (PI), Irgacure D-2959 (2-Hydroxy-4′-(2-hydroxyethoxy)-2- methylpropiophenone) was added to 1000 µl DPBS with the concentration of 1% (w/v) and dissolved in 80°C in a thermoshaker. Afterwards, the PI solution was added to GelMA with 0.5 % (w/v) concentration. To completely dissolve the GelMa, the solution was incubated in 80℃ for 60 minutes.

2.2.5. Preparation of cells

Human umbilical vein endothelial cell (HUVEC) and SH-SY5Y (neuroblastoma) cell lines were used in this experiment. The cells which were stored in liquid nitrogen were thawed in passages 19 and 17 respectively in a 37°C water bath. The medium was prepared using Dulbecco's Modified Eagle Medium (Gibco, Waltham, USA), 10% fetal bovine serum (Gibco, Waltham, USA), 1% penicillin streptomycin (Gibco, Waltham, USA), 1% L-Glutamine (Gibco, Waltham, USA) and 1% sodium pyruvate (Gibco, Waltham, USA). Cells were seeded at T75 cell culture flasks and incubated at 95% humidity, 5% CO2 and 37℃. Cells were subcultured once prior viability tests.

2.2.6. UV curing of GelMA and cell seeding inside microfluidic chip

OmniCure UV light spot curing source was used to cure the GelMA solution. The UV light spot curing source got its own probe and probe holder. The cells were added to the solution and injected inside of the middle channel of the microfluidic chip. Then UV light was applied with the parameters given at (Table 2.1).

Table 2.1: UV Curing parameters for the hydrogel fabrication inside of the microfluidic chip.

Variable	Value
UV Exposure Duration	50 seconds
Microchip distance from the UV Source	60 mm
Irradiance Level	6.25 W/cm^2
GelMA Concentration	10%
Photoinitiator Concentration	1%
Cell Concentration	$5*10^5/ml$

The top channel of the microfluidic chip was filled with HUVEC and medium prepared at the cell preparation phase. HUVEC was seeded at the top channel which got around 12µl volume. Afterwards, the bottom channel was filled with medium without any cells. After filling the channels, the microchip was put inside of a staining jar and incubated for 20-30 minutes with bottom channel at top, to accelerate medium flow through the middle channel. Afterwards, the chip was rotated upside down to promote cell movement towards the gel, and staining jar is filled with 4ml of distilled water to keep interior of the staining jar humid. The cell culture medium was renewed every day.

2.2.7. Preparing GelMA hydrogels for cell viability analysis

Three cell types, HUVEC, SH-SHY5Y and co-cultured HUVEC and SH-SHY5Y cells were encapsulated into GelMA inside well plates for three times. For this process, firstly, TMSPMA coated coverslips were placed inside of well plates prior polymerization to increase the surface adhesion. After placing the coverslip, cells and pre polymer solution were applied onto surface of the coverslips. Afterwards Same UV parameters at Table 2.1 were followed for photopolymerization and gels were manufactured as seen at (Figure 2.6).

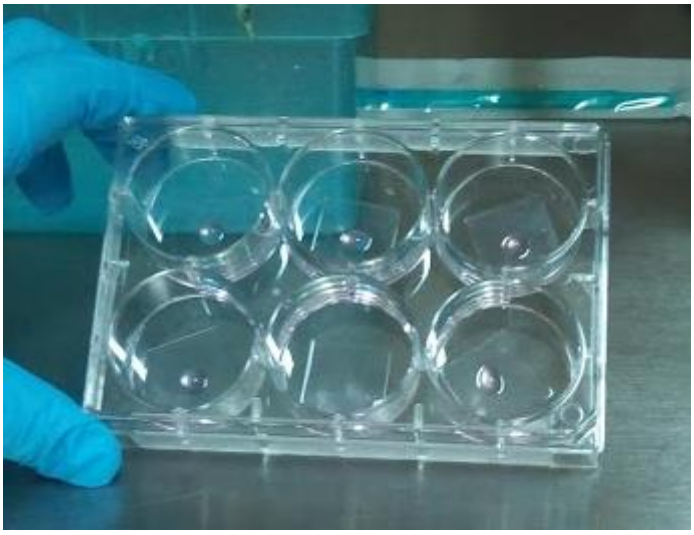


Figure 2.6: Photopolymerized GelMA hydrogels inside of a well plate.

This step was repeated 3 times for each cell types, for days 0,1,4 and 7, making 36 cell-encapsulated GelMA hydrogels in total.

2.2.8. Cell viability assay

The viability of cell encapsulated GelMA structures were measured using alamarBlue viability assay (ThermoFisher Scientific, USA). HUVEC, SH-SHY5Y and co-cultured HUVEC/ SH-SHY5Y encapsulated GelMA hydrogels were scraped from the well plates with using cell scraper and placed in a 96-well microplate one by one. This process was repeated for day 0,1,4 and 7 respectively to measure the viability values for those durations. After cell encapsulated hydrogel structures were seeded into microplates, 90 µL cell medium were added to each well. Medium was changed every day. 5 hours prior to getting viability results, cell mediums in each plate was refreshed and 10 µL of alamarBlue were added to plates, making total 100 µL cell medium and alamarBlue solution in each well. After five hours, viability results were measured using microplate reader with using absorbance value of 595 nm.

2.2.9. Atomic force microscopy analysis of GelMA hydrogels

Atomic Force Microscopy (AFM) was used to analyze the surface properties of the GelMA in hydrogel form. AFM was used in non-contact mode and its physical

properties were observed with using Nanosurf Flex-Axiom in Izmir Katip Çelebi University.

2.2.10. Viability imaging of encapsulated cells inside of microfluidic chip

Calcein AM and Propidium Iodide (PI) was used to locate the dead and live cells inside of the hydrogel. To analyze the viability of day 0, 1, 4 and 7 HUVEC and SH-SHY5Y cells were co cultured and encapsulated inside of the GelMA hydrogel in middle channel of the microchips. Both top and bottom channels were filled with medium with serum and incubated at 37 ℃, 5% CO2 and 95% humidity, mediums were refreshed every day. Prior to imaging, mediums in both top and bottom channels were removed and replaced with Calcein AM and PI respectively. Unlike the conventional protocols, both Calcein AM and PI were diluted 5 μM, higher than usual ratio, as we observed higher brightness with this dilution rate in our experiments. As gravitational force increases the amount of liquid passing the permeable surface of the 3D matrix from the top channel while microchip is positioned horizontally inside of the staining jar, effect of gravitational force should be minimized to distribute Calcein AM and PI as homogenously as possible. For this purpose, after both channels were loaded with Calcein AM and PI, in every 15 minutes positions of top and bottom channels were swapped horizontally by rotating the microchip 180 degrees as described at (Figure 2.7).

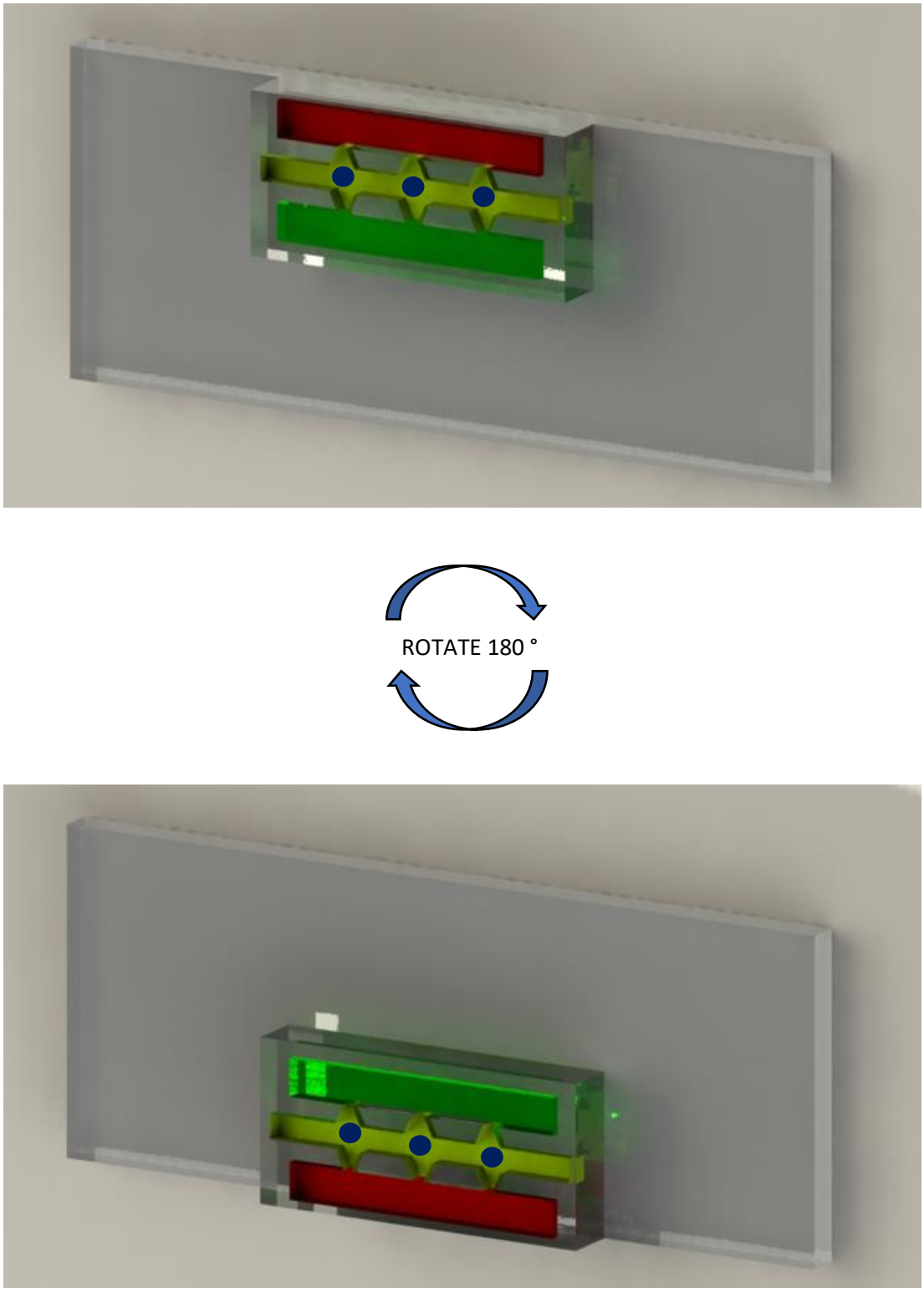


Figure 2.7: CAD drawing of the process used to distribute Calcein AM and PI as homogenously as possible inside of a staining jar. Green color refers to Calcein AM, Red color refers to PI. Yellow color refers to co-culture inside of GelMA. Blue dot refers to ROI spots where viability values were taken.

This process was repeated four times for total 1 hour. Afterwards, fluorescence imaging was performed using Olympus BX51 fluorescence microscope (ThermoFisher Scientific, USA). Images were taken from the center of the middle channel to get the result from the most equally distributed region of Calcein AM / PI. (Figure 2.8) shows the overall distribution of Calcein AM / PI over channels.

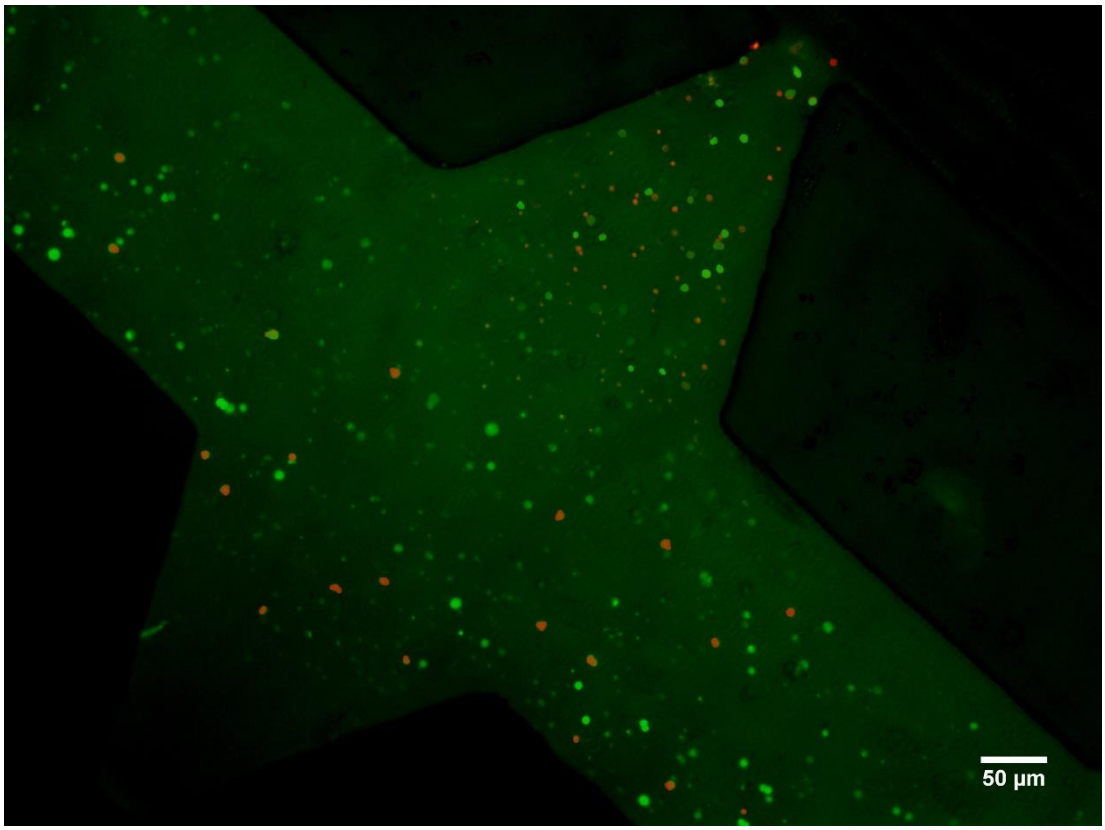


Figure 2.8: Distribution of Calcein AM/PI marked cells inside of the microfluidic chips.

2.2.11. Swelling test

It is vital for a hydrogel to have ideal swellinging consistency as encapsulated cells inside needs continious nutrition to become viable for long durations. Compared to GelMA hydrogel built inside a microfluidic chip, GelMA hydrogels built on conventional platforms such as petri dishes have ability to swell the medium faster as they have higher surface area in contact with medium and other nutrients. For this reason, it is important to confirm liquids in both top and bottom channels successfully get swallowed by the GelMA hydrogel in medium channel and diffuse efficiently

overnight. To observe the permability of the GelMA hydrogel visually inside of the microfluidic chip, trypan blue was used. Cell encapsulated GelMA hydrogel was built inside of the middle channel of microfluidic chip and both top and bottom channels were filled with medium and the microchips were incubated at 37 °C, 5% CO_2 and 95% humidity. Mediums were refreshed everyday. At day 1 and 7, mediums at both channels were removed and channels were replaced with trypan blue and left for overnight at incubation. Diagram at (Figure 2.9) visualizes the preparation process of swelling test.

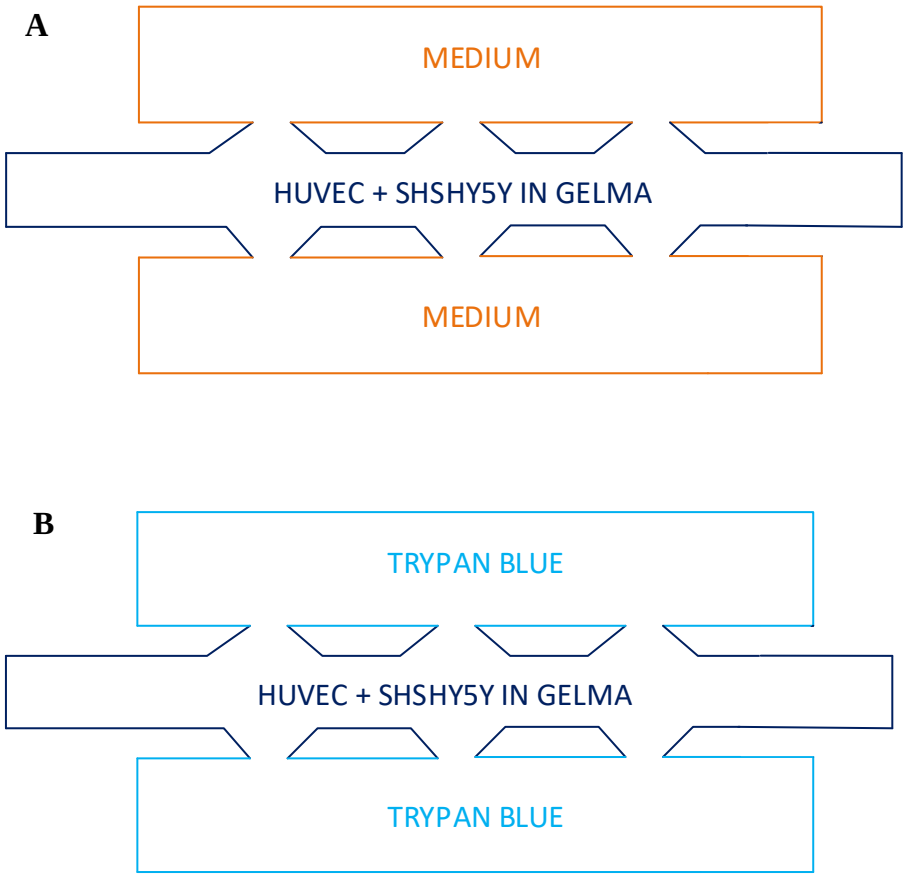


Figure 2.9: Diagram of the microchip after mediums were removed from top and bottom channels and replaced with trypan blue. A) Initial state of the microfluidic chip prior the experiment. B) Experiment setup for the overnight period.

After this process, images of the microchips were taken respectively and the permability of the hydrogel was observed visually.

2.2.12. Functional immunofluorescent staining

Fluorescent cell trackers were used to identify cell types and track their movements. Two different types of immunofluorescence dyes were used to stain SH-SHY5Y cells encapsulated inside of GelMA at the middle channel, and HUVEC, which were seeded inside of the top channel. To stain SH-SHY5Y cells CellTracker Green CMFDA dye was used and Cell tracker Red CMTPX was used to stain HUVEC. Both dyes were purchased from ThermoFisher Scientific, USA. Lyophilized dyes were dissolved in DMSO to the final concentration of 10 mM, and solution was diluted in 10 μM serum free medium. Then working solution was warmed to 37°C before using.

Two different protocols were used for two different types of cells. Staining protocol for cells in suspension, and staining protocol for adherent cells. For SH-SHY5Y, cells had to be dyed before the UV photopolymerization process as protocol for adherent cells require medium change which is not efficient for encapsulated cells. Before SH-SHY5Y cells were prepared for photopolymerization, they were centrifuged for 5 minutes and the supernatant was aspirated. Working solution was used to resuspend the cells, and cells are incubated at their growth environment, 37 °C, 5% CO_2 and 95% humidity for 30 minutes. Cells were centrifuged afterwards and working solution (Green CMFDA) was replaced with the growth medium and photopolymerization process began.

For HUVEC, at day 0, the growth medium inside of the microchip was removed and replaced with the working solution (Red CMTPX). Afterwards, cells were incubated inside of staining jar at 37 ℃, 5% CO_2 and 95% humidity for 30 minutes. Working solution was removed and replaced with a fresh medium with supplements. The fluorescent images were captured with Olympus BX51 fluorescence microscope, stacked and analyzed with using Image J software.

3. RESULTS

3.1. H^1 NMR Analysis of GelMA

In this process, metacrylation of GelMA was characterized using H^1 NMR technique. Methacrylamide groups (5.59 ppm and 5.8 ppm) and gelatin aromatic residues (7.4 ppm) were observed. Those results show the methacrylation process of gelatin was successful [23]. (Figure 3.1) and (Figure 3.2) shows the H^1NMR analysis results of gelatin and GelMA.

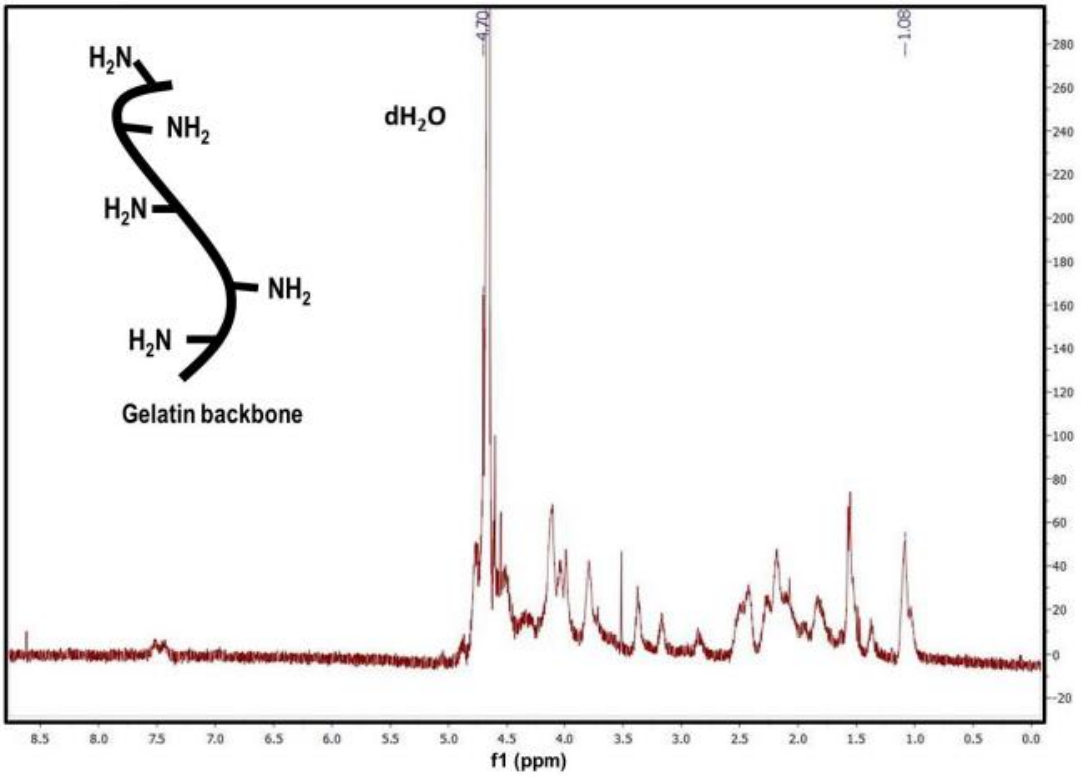


Figure 3.1: Gelatin H1 NMR Characterization Results

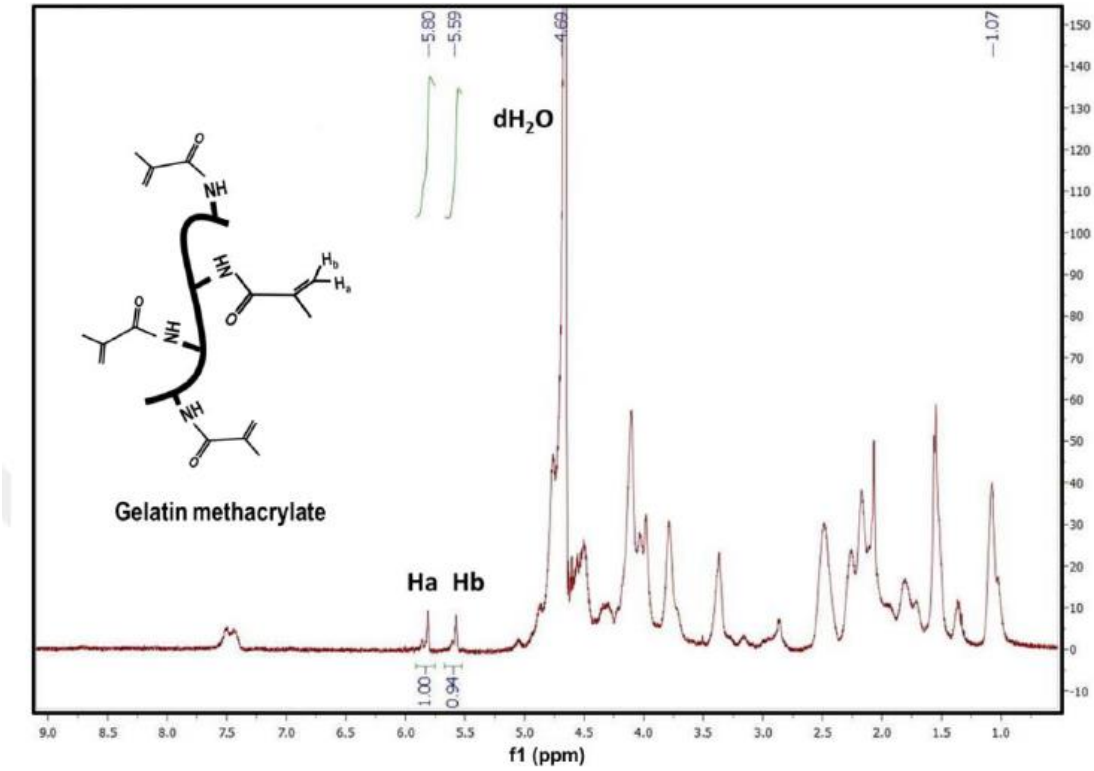


Figure 3.2: Gelatin Methacrylate H1 NMR Characterization Results

3.2. Fourier Transform Infrared Spectroscopy Analysis of GelMA

In order to confirm the synthesized GelMA had desired compounds in its structure, Fourier Transform Infrared Spectroscopy (FITR) analysis was made. The hydrogel spectra showed a broad peak around 3300 cm^{-1}, the peak around 1600 cm^{-1} is caused by C=0 stretching groups. Smaller peak around 1500-1550 refers to C-N-H structure. Overall, the FITR spectra values seen at (Figure 3.3) correlates with previously analyzed values of GelMA, which shows the synthesized GelMA have desired values and chemical structure [54].

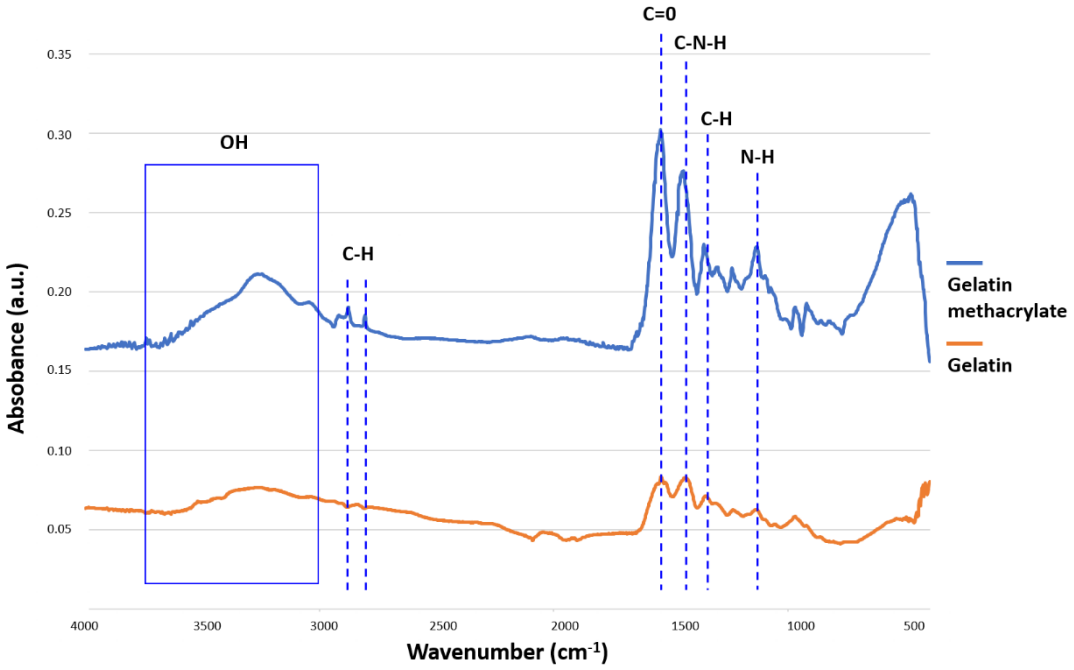


Figure 3.3: FITR spectra of GelMA and Gelatin

3.3. Field Emission Scanning Electron Microscopy Imaging of GelMA

Field Emission Scanning Electron Microscopy (FESEM) images of lyophilized and hydrogel form GelMA were taken to observe the surface morphology of both lyophilized and hydrogel form GelMA. Lyophilized GelMA showed a patterned surface morphology with pores up to around 60 μm. In contrast, hydrogel form GelMA showed irregular pattern of porous surface morphology, having more pores overall with lesser diameters, seen at surface characterization images at (Figure 3.4) and (Figure 3.5).

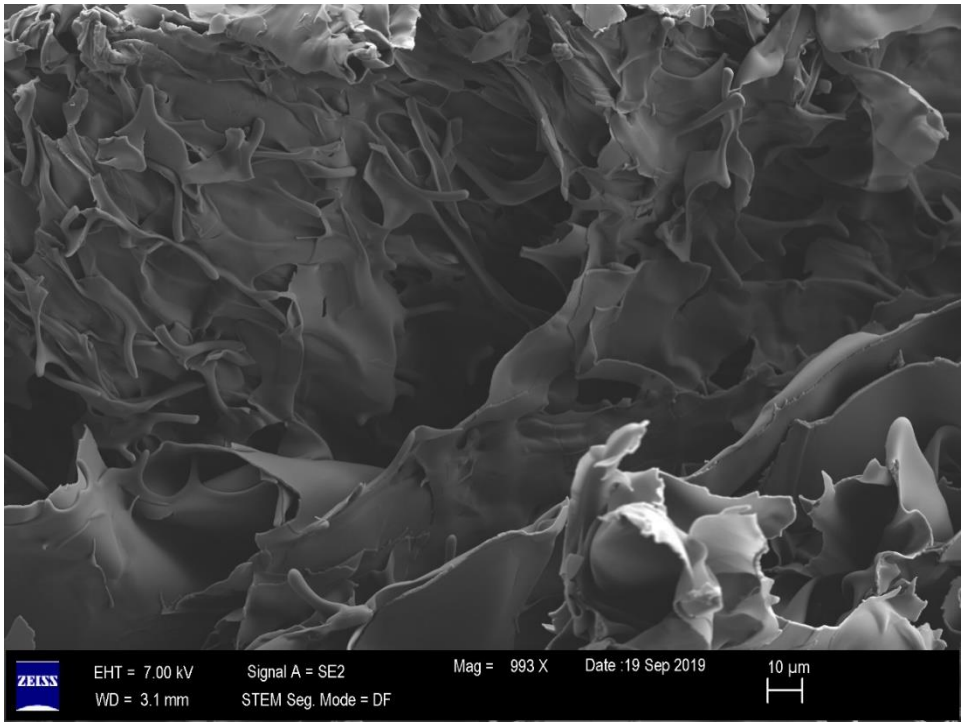


Figure 3.4: FESEM image of lyophilized GelMA surface.

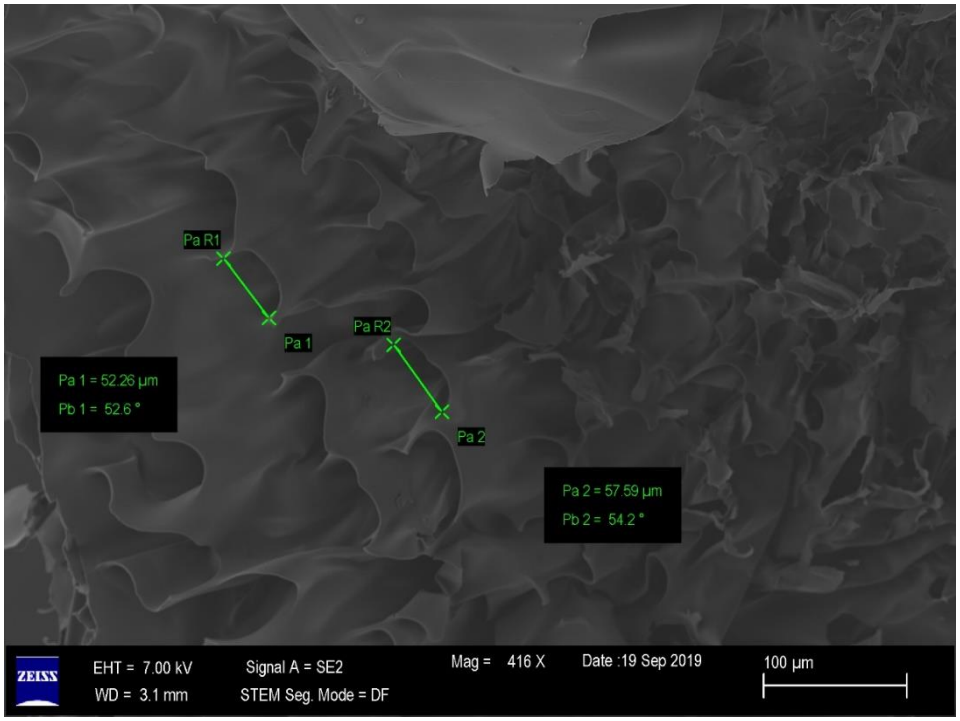


Figure 3.5: FESEM image of the lyophilized GelMA surface with pore sizes shown.

3.4. Atomic Force Microscopy Analysis of GelMA Hydrogel

Atomic force Microscopy (AFM) was used to observe the structural properties of GelMA hydrogel. Non-contact mode was used at scanning and analysis values were shown at (Figure 3.6).

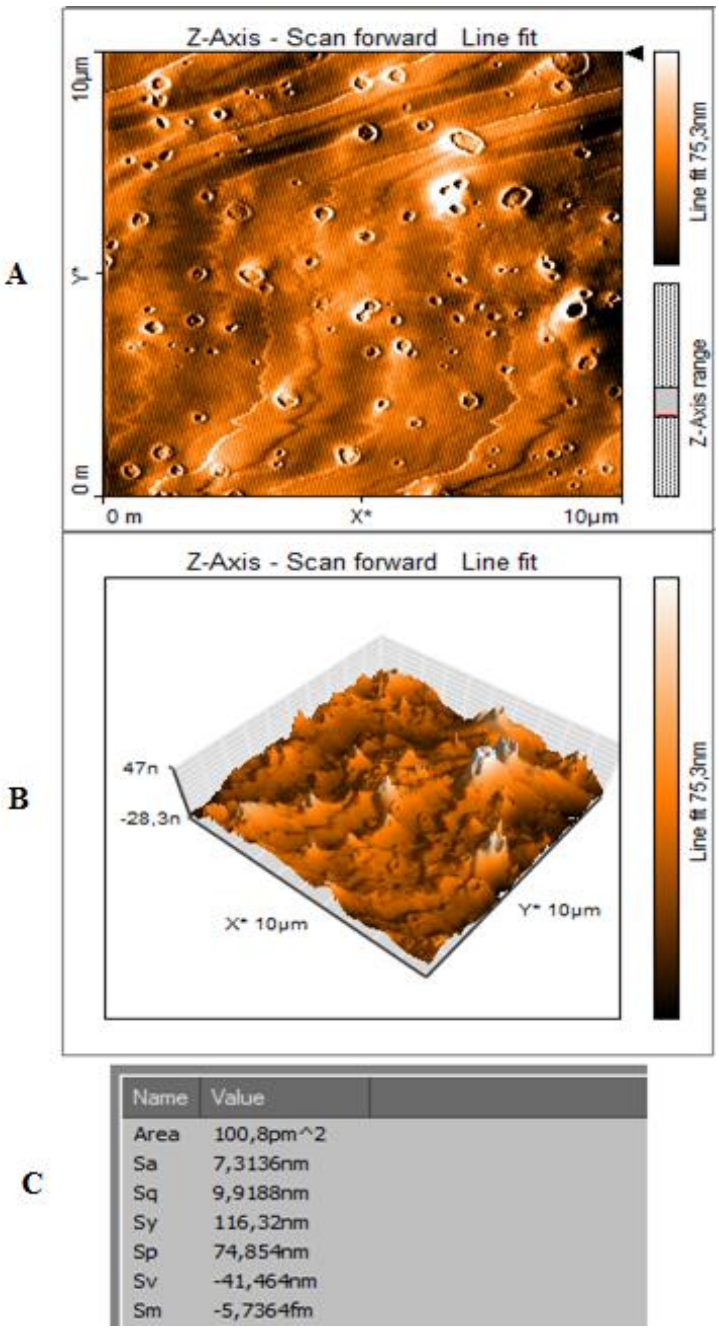


Figure 3.6: Non-Contact mode AFM scanning of the surface of GelMA hydrogel. (A) 2D image of the hydrogel surface. (B) 3D image of the hydrogel surface. (C) Analysis parameters and measured results.

3.5. Cell Viability Test

The viability of the encapsulated cells outside of microfluidic platform were studied. In this experiment, HUVEC, SH-SHY5Y and co-cultured HUVEC & SH-SHY5Y cells were encapsulated inside GelMA hydrogels on TMSPMA coated surface. For day 0, 1, 4 and 7 each, absorbance ratios of three different cell types were gathered, normalized and quantitatively analyzed. Cell viability ratios of day 0, 1, 4 and 7 were 100%, 77%, 80% and 82% for HUVEC cells, 100%, 80%, 82% and 85% for SH-SHY5Y cells and 100%, 70%, 85% and 91% for co-cultured cells respectively as seen at (Figure 3.7).

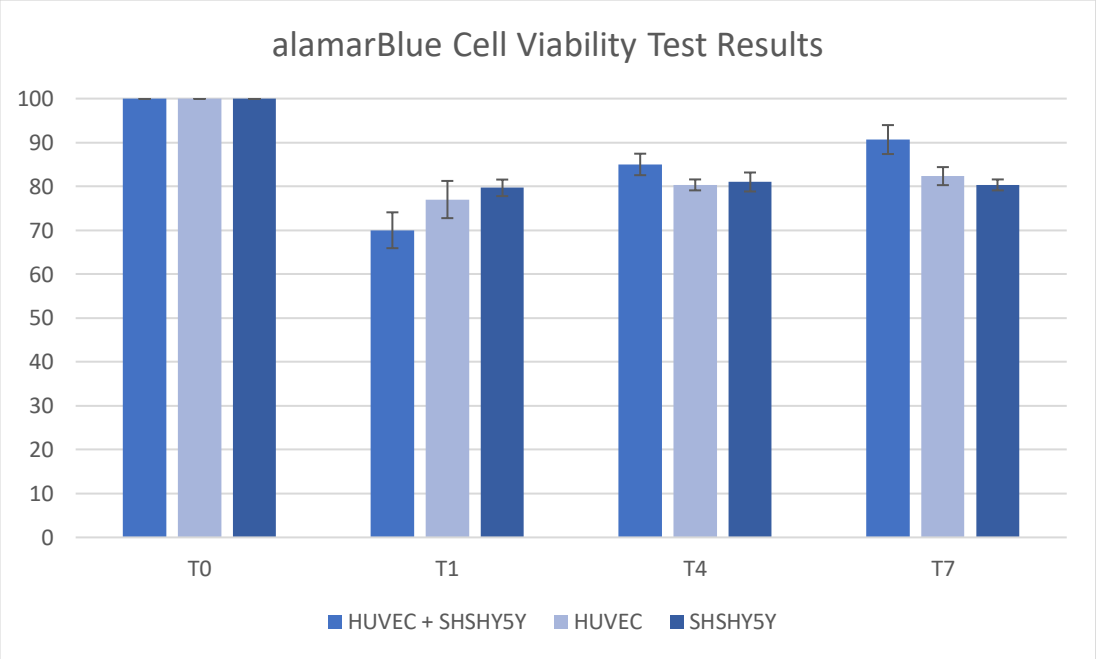


Figure 3.7: The cell viability test results of endothelial and neuroblastoma cells encapsulated into GelMA hydrogel. (n=3)

Value of the overall cell viability for those four days were 100%, 73%, 82.5% and 88%, which shows viability values of HUVEC and SH-SHY5Y cells encapsulated inside of GelMA hydrogels were promising.

3.6. Swelling Test of the GelMA Hydrogel Inside of the Chip

To visually confirm whether the hydrogel inside of the microchip was capable of swelling enough liquid in overnight period for a week, GelMA hydrogels in two microchips were tested at day 1 and 7 respectively. Microfluidic environment containing co-cultured HUVEC and SH-SHY5Y cells were supplied with fresh medium every day and before the test, mediums from both top and bottom channels were removed and replaced with trypan blue as seen at (Figure 3.8).

Figure 3.8: The initial state of the microfluidic chip at the beginning of the experiment.

To visually confirm GelMA hydrogel is capable of swallowing liquid with same efficiency at day 1 and day 7, the expected result was medium channel swallowing denser amount of liquid compared to other channels, resulting in darker blue color, and diffusing efficiently, resulting no any “white space” inside of middle channel. As seen at (Figure 3.9) both hydrogels were able to swallow trypan blue efficiently in overnight, which shows hydrogels have the capacity of swallowing enough medium in overnight period for at least 7 days.

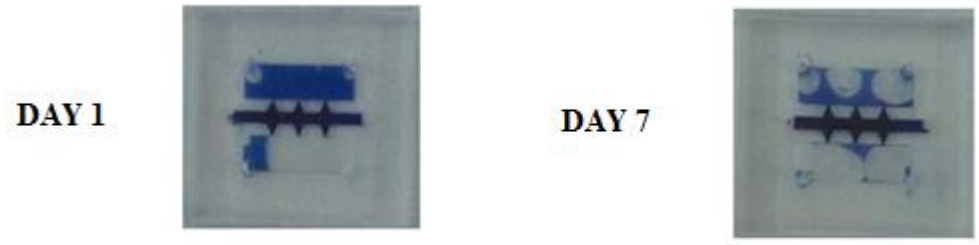


Figure 3.9: Day 1 and day 7 images of the microfluidic chip after incubating with trypan blue overnight.

3.7. Cell Viability Imaging of GelMA Inside of the Microfluidic Chip

To analyze the cell viability of co-cultured HUVEC and SH-SHY5Y cells encapsuled in GelMA hydrogel inside of microfluidic chip, Calcein AM and Propidium Iodide (PI) was used. Viability images of day 0, 1, 4 and 7 were taken as seen at (Figure 3.10).

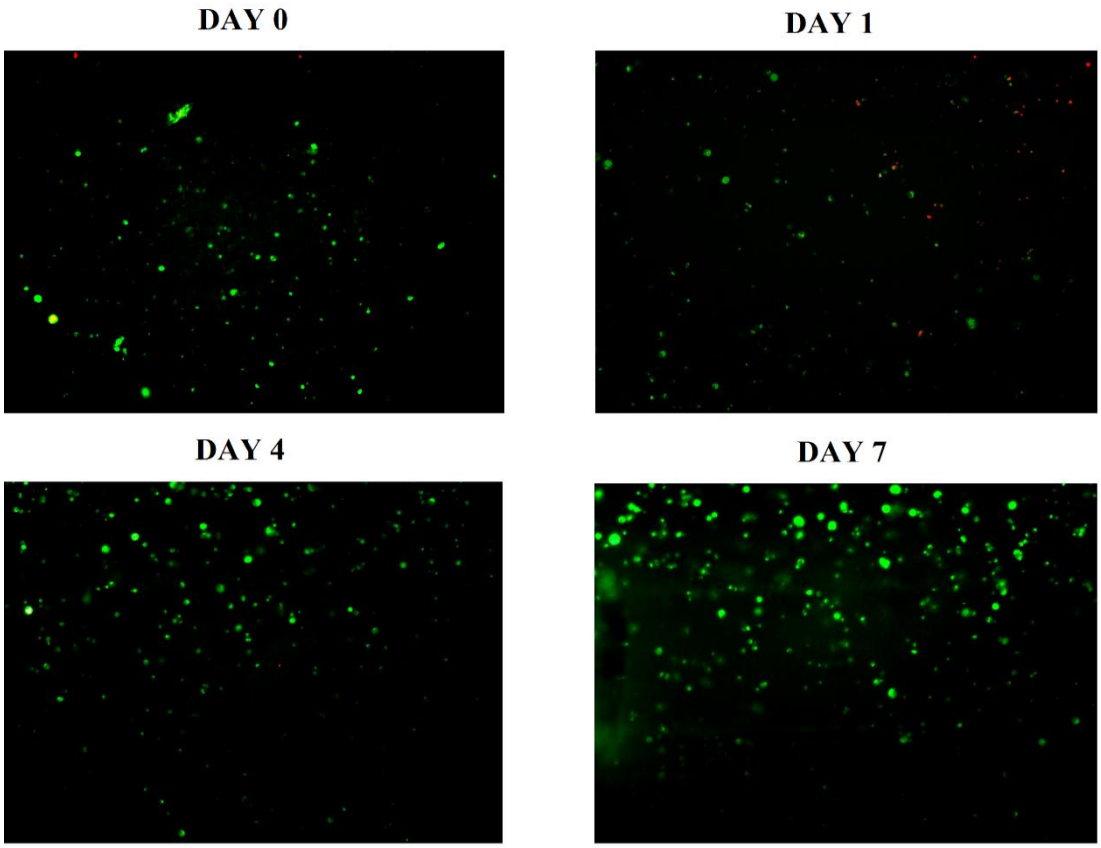


Figure 3.10: The Cell Viability Images of co-cultured HUVEC and SH-SHY5Y cells inside of GelMA hydrogel.

Co-cultured cells inside of GelMA hydrogel in microfluidic chips showed viability results of 83%, 75%, 84% and 89% for day 0, 1, 4 and 7 respectively as seen at (Figure 3.11).

.

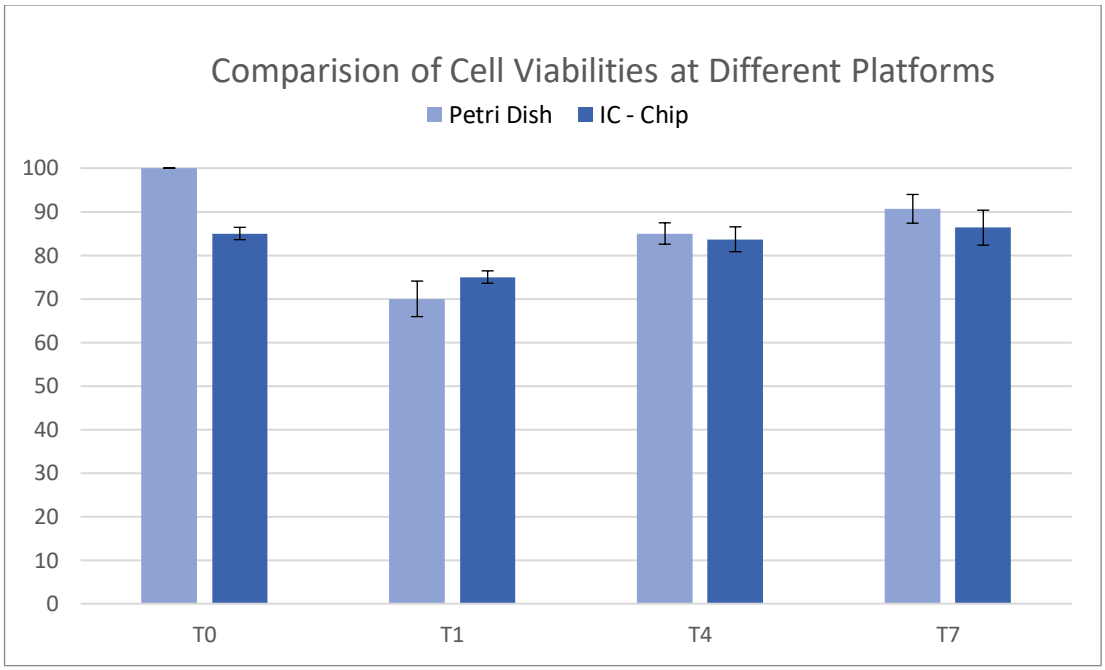


Figure 3.11: Comparison of encapsulated co-cultured cells inside of GelMA hydrogel in different platforms. (n=3)

As we examine the data further, we can see a linear relationship between viability values for both platforms at the data values shown at (Figure 3.12).

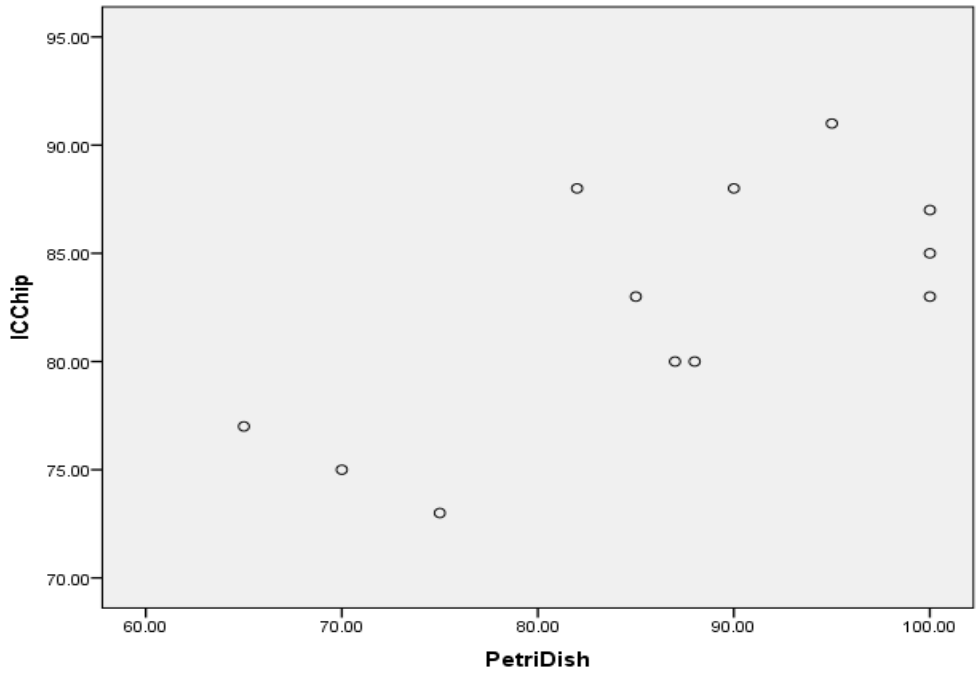


Figure 3.12: Cellular viability values of two platforms.

Even though linear relationship was visible, a correlation analysis was conducted to see if those values had exact relationship. Before conducting a correlation analysis, a normality test had to be done in advance. As dataset was small (n = 3 for each day), Shapiro-Wilk significance values at (Table 3.1) had to be higher than 0.05.

Table 3.1: Normality test results.

	Kolmogorov-Smirnov			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
IC-Chip	.121	12	.200	.963	12	.824
Petri Dish	.126	12	.200	.926	12	.342

As it's shown at (Table 3.1), significance value is greater than 0.05 therefore we can say data follows a normal distribution, which allows us to apply a parametric statistical model for this dataset.

When we apply correlation analysis for the cellular viability values, results were as shown at (Table 3.2) and (Table 3.3).

Table 3.2: Mean and standard deviation values of cellular viability of co-cultures in different platforms.

	Mean	Std Deviation	N
IC-Chip	82.500	5.632	12
Petri Dish	86.416	11.766	12

Table 3.3: Pearson correlation values of cellular viability of co-cultures in different platforms.

		IC-Chip	Petri Dish
IC-Chip	Pearson Correlation	1	.704
	Sig. (2-tailed)		.011
	N	12	12
Petri Dish	Pearson Correlation	.704	1
	Sig. (2-tailed)	.011	
	N	12	12

According to results, we can say that there is a positive linear relationship at the 0.05 significant with 70,4% correlation.

Therefore, we can say there is no significant positive or negative cellular viability difference between encapsulating cells inside of GelMA hydrogels outside or inside of a microfluidic chip as well as cell viability values correlate with each other.

3.8. Functional Immunostaining of Cells

Two types of fluorescence cell dyes were used to track the localization of cells. CellTracker™ Red CMTPX dye was used to stain HUVEC, CellTracker™ Green CMFDA dye was used to stain SH-SHY5Y cells. In addition to localization of cells, those dyes were also used to observe the structural stability of the gel in the microfluidic chip through days.

The first intersection points from each side were observed in this process. Intersection point between the first bottom and middle channel was used as control point as bottom channel only includes growth medium whereas top channel was consisting HUVEC and growth medium as described at (Figure 3.13).

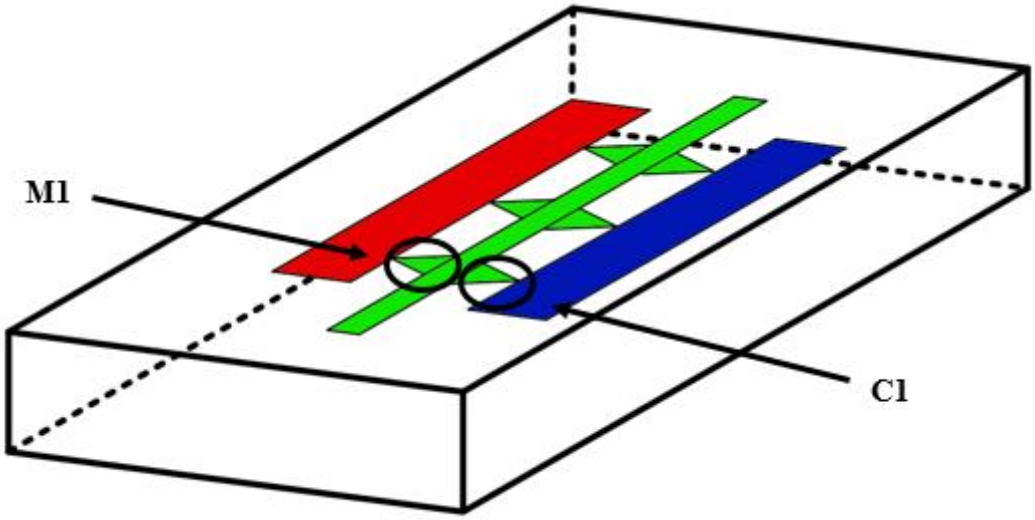


Figure 3.13: Experimental setup to observe cell migration inside of the GelMA structure. M1 and C1 refers to region of interests where images were taken. Channel painted with red color contains HUVEC and growth medium whereas green channel contains SH-SHY5Y and GelMA. Blue channel contains growth medium which was used as control medium.

Intersection point between the top and middle channel was used to observe the cell localization through time. Those two intersection points was referred as C1 and M1 respectively as seen at (Figure 3.14).

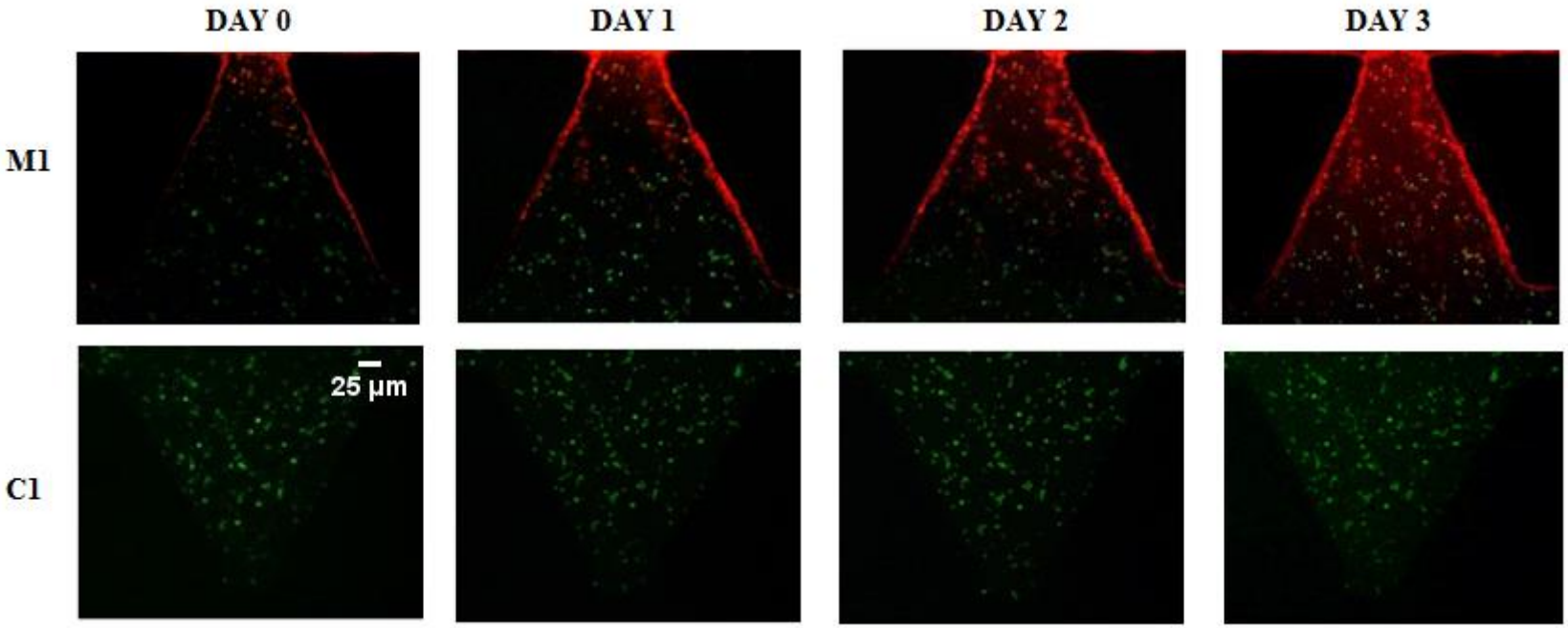


Figure 3.14: Immunoflourescence images of selected ROIs on IC-Chip through 72 hours. HUVEC was dyed with red immunoflourescence dye whereas SH-SHY5Y was dyed with green dye.

Besides the localization of HUVEC and SHSHY5Y cells inside of IC-Chip, structural stability of GelMA hydrogel inside of the chip was also observed. 6 intersection points of three different chips were investigated daily using fluorescence microscopy, and structural stability of GelMA hydrogel at those points was observed. For three hydrogels all of them lasted longer than 28 days, one structurally deforming at day 29, and others at day 34 and 32 respectively. (Figure 3.15) shows a deformed GelMA structure inside of the microfluidic chip.

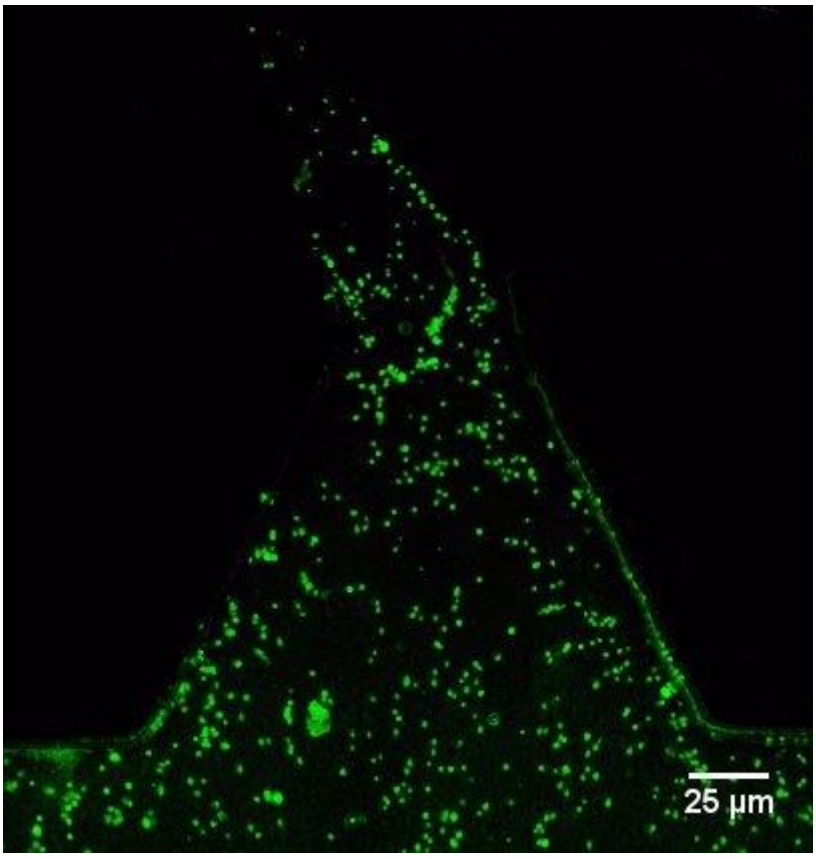


Figure 3.15: Fluorescence microscopy image of a deformed GelMA hydrogel inside of IC-Chip

4. DISCUSSION

In tissue engineering, modeling a structurally stable extracellular matrix that is capable of mimicking the natural extracellular environment of the cell efficiently has a vital importance. For this purpose, GelMA has shown structural stability and ability to intimate the natural environment of the cell accurately as it is being used at many tissue engineering applications. However, with using biocompatible gels, modeling cellular migration and invasion with conventional methods using matrigels, or with tools like well plates are challenging compared to using photopolymerization on microfluidic systems. In most applications on modeling a 3D cell culture environment inside a microfluidic chip, matrigels are being used. However, they have many drawbacks in manufacturing processes, such as complex and inconvenient procedures, like having to use ice inside of the biosafety cabinet while forming the gel and overall, they are less convenient and more expensive compared to photopolymerizable GelMA hydrogels. As PDMS is being used as the main material to manufacture microfluidic devices, those devices have excellent opacity, almost same level with glasses or petri dishes. This means producing a GelMA hydrogel using photopolymerzation on a microfluidic chip is a viable option for co culturing and modeling migration/invasion studies.

4.1. Hydrogel fabrication inside of the microfluidic chip

There are many desired properties to create hydrogels to encapsulate cells, one of those properties are shape and structure of the hydrogels. There are various strategies to create biocompatible hydrogels that are able to mimic to ECM efficiently, but options get narrow when producing hydrogels in desired shape with inner structures that are stable for long term. Expensive methods, such as 3D printing is one of the main strategies on this field. Even the printing resolution is getting higher and more precise, the process before printing is less convenient and more complex compared to UV

photopolymerization. For UV photopolymerization, process is rather easy, but the main concerns are the shape and the structural stability of hydrogel, especially in long term. As shown in this research, photopolymerization in microfluidic environment is able to increase the structural stability of the gel in long term while easily forming gel in a desired shape. On top of that, compared to strategies such as using matrigels or 3D printing strategies, UV photopolymerization is way faster compared to those, as this method only requires 20 - 60 seconds to build a gel.

4.2. Microfluidic applications for co-culture and migration studies

Migration and invasion studies are easier to model on microchip compared to conventional methods mainly because of the ability to control and manipulate the fluid flow inside the channels. For example, using the gravitational force to control the fluid flow inside of the channels by simply putting the microchip in a horizontal position while in incubation period promotes migration and invasion activities towards the direction of the gravitational force without needing extra protocols or environments such as transwell chambers or matrigels. Also compared to conventional models, mechanical forces applied to the hydrogel are way lesser as non-contact points organize structural stability of the hydrogels while intersection points let controlled activity of co-culturing processes. Mechanical forces are only applied in desired locations unlike conventional methods where mechanical forces such as shear stress are applied to wider surface area of the gel, which fastens the structural deformation process making the structure less viable for longer term.

Besides mechanical properties and stability of a photoinitiated GelMA hydrogel inside of a microfluidic chip, the cellular viability of the 3D matrix structure plays a vital role to conduct experiments in desired duration. As it is shown in this research, cellular viability inside the microfluidic IC-Chip was almost same with cellular viability outside of this platform. This means there are no major drawbacks of using GelMA hydrogels on this platform when cellular viability is considered.

In this research, it has been shown it is also possible to model a neural tissue using photopolymerizable GelMA inside of the microchip for both short and long term. In

addition to encapsulating neuronal cells such as neurons and astrocytes in a three-dimensional extracellular matrix, endothelial cells, co-cultured with neuronal cells inside of this structure can be used to create various neurovascular structures as a simple proof of concept creation. With using this model, also effects of drugs or various molecules on this cell encapsulated 3D environment can be tested with using those entities alongside seeded cells or only with medium at selected channels.

5. CONCLUSION

One of the most promising fields of tissue engineering is neural tissue modeling. Building accurate tissue models in three dimensional environments that are capable of mimicking their natural environments in vivo are the greatest challenges of neural tissue modeling as animal tests are either complex to build and understand or inaccurate for human tissue models. To overcome those difficulties, there are various neural tissue models to study those intra and extra cellular activities. With the increasing amount of manufacturing methods and with the technological advancements, producing microfluidic devices became cheaper and easier compared to methods and materials used several years ago. For several years, neural tissue models are being developed on various microfluidic devices, however most of those models are inconvenient and takes longer time to model. In this research, the neural tissue model was developed in microchip using the photopolymerization method to form the hydrogel structure inside of the IC-Chip which is convenient, cheap and easy to use.

This study showed, it is possible to create a neural tissue model ready to use in a microchip in shorter time with simple methods using photopolymerizable GelMA model on microfluidic chips. On top of that with the ability of controlling the fluid flow, it is possible to follow and manipulate the localization of cells which are getting co-cultured. Also, with less amount of mechanical forces applied to the surface area of the hydrogel structure inside of the chip, the structural stability of the hydrogel lasts for more than 3 weeks which makes this method also suitable for long term studies. Finally, the cell viability tests results show cells are viable inside of the photopolymerized hydrogel at the UV exposure duration needed to build the gel structure inside of the microchip.

6. FUTURE PROSPECTS

In this study, structural properties and bioavailability of GelMA hydrogels were examined inside and outside of microfluidic chips with co-culturing a neurovascular tissue structure. Besides the neurovascular use of this setup, this environment is also suitable to model various types of cancer models and examine invasion and migration of various cancer or tumor cells. Also, this platform allows chemotaxis studies too, allowing chemoattractants to be used to promote migration of cells from the selected channels. As design of microfluidic devices let user to "mold" their hydrogel patterns easily without even needing surface coating or similar chemical or mechanical entities, with a well-designed microfluidic device, patterning a desired shape and sustaining the cell environment in a matrix can be easier compared to alternative methods.

Our experiment proved that photoinitiating GelMA hydrogel inside of a well- designed microfluidic chip, IC-Chip in this research, is a viable strategy to conduct co-culturing experiments as well as modeling invasion and migration events. This property can also be used on experimental studies on drug delivery and drug dosage optimization studies on various diseases. In addition, with ability to control the fluid flow inside of channels, studies on drug delivery speed and frequency might be conducted using this model.

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