Tampereen yliopisto

Lotta Isosaari

OPTIMIZING HYDROGELS FOR NEURO-VASCULAR CO-CULTURE STUDIES IN MICROFLUIDIC DEVICES

Faculty of Medicine and Health Technology
Bachelor's Thesis
April 2021

ABSTRACT

Lotta Isosaari: Optimizing hydrogels for neuro-vascular co-culture studies in microfluidic devices
Bachelor's Thesis
Tampere University
Degree Programme in Biotechnology and Biomedical Engineering
April 2021

Neuronal and vascular networks cover the whole human body and are essential for the normal functions of tissues. Nervous systems rely on electrochemical signals to transfer information and control the homeostasis of human body, while the vascular network provides oxygen and nutrients for the cells in all tissues. Since these two network systems are crucial for the normal functions of tissues, it is important to understand their roles and interactions in tissue development, maintenance of homeostasis and in different pathological conditions. Thus, neuro-vascular *in vitro* models are needed for obtaining a better understanding about the relationship between these two networks.

Because the two-dimensional (2D) models are not good at mimicking the actual environment the cells have *in vivo*, three-dimensional (3D) models are needed for more realistic tissue models. Hydrogels are commonly used for 3D cell cultures. Those are three-dimensional networks that have a major water absorption capacity. In this thesis, hydrogels and cell culture media were optimized for human induced pluripotent stem cell (hiPSC) -derived neural progenitor cells. The aim was to see how different cell culture media affect hydrogels and neuronal cell growth. This information can later be applied to neuro-vascular co-culture studies.

In the study, culturing hiPSC-derived neural progenitor cells was tested in 1) collagen I hydrogel, 2) fibrin hydrogel and 3) collagen I–fibrin hydrogel. The cells were seeded inside the three different hydrogels in 24-well-plate format. In collagen I and collagen I–fibrin hydrogels cells were seeded also in commercial AIM Biotech 3D Cell Culture Chips. For the cell cultures in AIM Biotech chips, endothelial cell growth medium-2 (EGM-2), neural maturation media (NMM) and 1:1 mixture medium of EGM-2 and NMM were tested. The cell cultures were maintained for up to 14 days. The growth of the cells and the appearance of the hydrogel were being observed during the culturing period.

The results showed that collagen I hydrogel stayed intact in both 24-well-plate and AIM Biotech chip in NMM for 14 days. However, used cell culture media affects collagen I hydrogel integrity and collagen I hydrogel shrunk in AIM Biotech chip in EGM-2 medium and 1:1 mixture medium. Fibrin hydrogel showed strong degradation in NMM in 24-well-plate and the experiments were not continued in AIM Biotech chips. Collagen I–fibrin hydrogel showed degradation and shrinkage during the culturing period in NMM in 24-well-plate. The shrinkage was major in AIM Biotech chip cultures and it was observed in cultures in all three media. In addition to affecting hydrogel integrity, used cell culture media affected also neuronal cell growth. In NMM, the growth of the cells was typical for neuronal cells. EGM-2 medium changed the growth of the cells. In 1:1 mixture medium there was also some cells that expressed untypical growth.

The information gained from hydrogel optimization performed in this study serves as a basis for finding optimal hydrogel for 3D neuro-vascular co-cultures in microfluidic cell culture device. More studies need to be performed with collagen I hydrogel and collagen I–fibrin hydrogel to find an optimal hydrogel for neuro-vascular co-cultures.

Keywords: hydrogels, microfluidic system, neuro-vascular interactions, 3D cell culture

TIIVISTELMÄ

Lotta Isosaari: Neuro–vaskulaari-yhteisviljelmätutkimuksiin soveltuvan hydrogeelin optimointi mikrofluidistisissa kudossiruissa
Kandidaatintutkielma
Tampereen yliopisto
Bioteknologian ja biolääketieteen tekniikan tutkinto-ohjelma
Huhtikuu 2021

Hermo- ja verisuoniverkostot kattavat koko ihmiskehon ja ovat välttämättömiä kudosten normaalille toiminnalle. Hermosolujen välinen viestintä solulta toiselle perustuu sähkökemiallisiin signaaleihin. Näiden signaaleiden avulla solut välittävät informaatiota ja ylläpitävät homeostasiaa ihmiskehossa. Verisuonet taas tarjoavat happea ja ravinteita soluille kaikissa elimistön kudoksissa, jonka lisäksi ne osallistuvat immuunipuolustukseen ja kudosten korjausprosesseihin. Koska nämä kaksi verkostoa ovat välttämättömiä elimistön normaalille toiminnalle, on tärkeää ymmärtää niiden keskinäiset roolit ja vuorovaikutukset kudosten kehityksessä, homeostasian ylläpidossa sekä erilaisissa patologisissa tiloissa. Neuro-vaskulaari *in vitro* -malleja tarvitaan näiden vuorovaikutusten tutkimiseen ja ymmärtämiseen.

2D-kudosmallit eivät jäljittele hyvin solujen todellista *in vivo* -ympäristöä, minkä vuoksi tarvitaan 3D-malleja realistisempien kudosmallien luomiseksi. Hydrogeelit ovat materiaaleja, joita käytetään yleisesti 3D-soluviljelmissä. Ne ovat kolmiulotteisia verkkoja, jotka pystyvät sitomaan itseensä suuren määrän vettä. Tässä tutkielmassa hydrogeelejä ja soluviljely mediumeja optimoitiin ihmisen indusoiduista pluripotenteista kantasoluista (human induced pluripotent stem cell, hiPSC) erilaistetuille hermosolujen kantasoluille mikrofluidistisissa kudossiruissa. Tutkielman tarkoituksena oli selvittää, kuinka hydrogeelit ja solut reagoivat erilaisiin soluviljely mediumeihin. Tutkielmasta saatavaa informaatiota voidaan myöhemmin hyödyntää neuro–vaskulaari-yhteisviljelmätutkimuksiin.

Tutkimuksessa hiPSC-erilaistettujen hermosolujen kantasolujen viljelyä testattiin erilaisissa hydrogeeleissä. Käytetyt hydrogeelit olivat 1) kollageeni I -hydrogeeli, 2) fibriinihydrogeeli ja 3) kollageeni I–fibriini -hydrogeeli. Solujen viljelyä testattiin kaikissa kolmessa hydrogeelissä 24-kuoppalevyllä. Kollageeni I -ja kollageeni 1–fibriini -hydrogeeleissä solujen viljelyä testattiin myös kaupallisessa AIM Biotech:n 3D-kudossirussa. AIM Biotech:n kudossiruissa oleville näytteille testattiin myös kolmea eri soluviljely mediumia, jotka olivat 1) endoteelisolujen kasvumedium (EGM-2), 2) neuraalinen maturaatio medium (NMM) ja 3) 1:1 sekoitusmedium, joka koostui EGM-2 -mediumista ja NMM-mediumista. Soluviljelmiä ylläpidettiin tutkimuksessa korkeintaan 14 päivän ajan. Tämän ajanjakson aikana solujen kasvua ja hydrogeelien ulkonäköä tarkasteltiin.

Tutkimuksessa osoitettiin, että kollageeni I -hydrogeeli pysyy ehjänä sekä 24-kuoppalevyllä että AIM Biotech:n kudossiruissa NMM-mediumissa 14 päivän ajan. Käytetyt soluviljely mediumit vaikuttivat kuitenkin kollageeni I -hydrogeelin eheyteen, sillä EGM-2 –mediumissa ja 1:1 sekoitusmediumissa hydrogeeleissä oli havaittavissa kutistumista AIM Biotech:n kudossiruissa. Fibriinihydrogeelit hajosivat nopeasti NMM-mediumissa 24-kuoppalevyillä, minkä vuoksi tutkimuksia ei jatkettu AIM Biotech:n kudossiruissa. Kutistumista havaittiin kollageeni I–fibriini -hydrogeelissä 24-kuoppalevyillä NMM-mediumissa sekä AIM Biotech:n kudossiruissa. AIM Biotech:n kudossiruissa hydrogeelin kutistuminen oli voimakkaampaa kuin 24-kuoppalevyllä, ja sitä havaittiin kaikissa kolmessa testatussa soluviljely mediumissa. Käytetty soluviljely medium vaikutti hydrogeelien eheyden lisäksi solujen kasvuun. NMM-mediumissa solujen kasvu oli tyypillistä hermosoluille. EGM-2 -medium muutti solujen kasvua epätyypilliseksi hermosoluille. Myös 1:1 sekoitusmediumissa oli havaittavissa jonkin verran epätyypillisesti kasvavia soluja.

Tutkimuksesta saatua informaatiota hydrogeelien optimoinnista hiPSC-erilaistetuille hermosolujen kantasoluille voidaan käyttää pohjana optimaalisen hydrogeelin löytämiselle 3D-neuro–vaskulaari-yhteisviljelmille mikrofluidistisissa kudossiruissa. Optimaalisen yhteisviljelmiin soveltuvan hydrogeelin löytämiseksi on suoritettava lisää tutkimuksia erityisesti kollageeni I -ja kollageeni I–fibriini -hydrogeeleillä.

Avainsanat: hydrogeelit, mikrofluidistiikka, neurovaskulaariset vuorovaikutukset, 3D-soluviljelmä

PREFACE

This thesis is a part of my Bachelor of Science studies at the Faculty of Medicine and Health Technology (MET) at Tampere University.

I would like to express my gratitude for Prof. Susanna Miettinen and Adj. Prof. Susanna Narkilahti for giving me the opportunity to work in their project and carry out my bachelor's thesis. Special thanks to my supervisors, PhD Hanna Vuorenpää and PhD Tiina Joki, for their great guidance and help. I also want to thank all the people in the Adult Stem Cell Group, Neuro Group, the Centre of Excellence in Body-On-a-Chip research community and in the MET-faculty whom I have worked with during this project. Last, I want to thank my family and friends for their support during the writing process.

Tampere, 23.04.2021

Lotta Isosaari

ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
ACA	ε-aminocaproic acid
BBB	blood-brain barrier
BDNF	brain-derived neurotropic factor
CNS	central nervous system
db-cAMP	dibutyryl-cyclicAMP
DPBS	Dulbecco's phosphate-buffered saline
ECM	extra cellular matrix
EBM-2	Endothelial Cell Growth Basal Medium-2
EGM-2	Endothelial Growth Medium -2
GDNF	glial-derived neurotropic factor
hiPSC	human induced pluripotent stem cell
MMPs	matrix metalloproteases
N3	neural maintenance media
NMM	neural maturation media
PBS	phosphate buffered saline
PDMS	polydimethylsiloxane
PNS	peripheral nervous system
VEGF	vascular endothelial growth factor

CONTENTS

1. INTRODUCTION

The nervous system is divided into central and peripheral nervous systems. The central nervous system (CNS) comprises the spinal cord and brain while the peripheral nervous system (PNS) comprises all the nerves outside the central nervous system. There are two main cell types in the nervous systems. Neurons form the neuronal network and are responsible for the neuronal communication and glial cells are the supporting cells for the neurons. Neural networks use electrochemical signals to transfer information and control the homeostasis of human body. (Stiles and Jernigan, 2010)

Vasculature is another network system in the body, formed by the blood vessels, which are all lined with endothelial cells. Blood vessels, arteries, veins and capillaries carry blood and circulate it through the body, delivering oxygen and nutrients for the cells in all tissues. They also have a role in immune system by carrying leukocytes in the blood stream (Ager, 2017). Endothelial cells act as a selective barrier, controlling the substances moving from blood stream to tissues and the other way around (Goddard and Iruela-Arispe, 2013). Blood vessels also play crucial role in the development, growth and repair of tissues.

Neurons and vascular structures are present in most of the various tissues that can be found in humans and therefore, it is important to understand their roles and interactions in maintaining the homeostasis and in tissue generation and development (Osaki et al., 2018). Understanding the normal interactions can also help to understand the mechanisms that underlie behind pathological conditions.

1.1 Hydrogels

In two-dimensional (2D) models, cells are cultured on a flat surface. These conventional culturing methods are still usable for many applications, but they do not present the realistic microenvironment that cells have *in vivo*. This is why three-dimensional (3D) models are essential for accurate modelling of interactions between neuronal and vascular networks. (Caliari and Burdick, 2016) The difference in the complexity of 2D and 3D cultures is demonstrated in Figure 1. Hydrogels are widely used tools for achieving the 3D environment for cell cultures. These hydrophilic gels are 3D networks that have a great water absorption capacity, which means that hydrogels can absorb fluids such as water into their network in large amounts (Zhang and Khademhosseini, 2017). Hydrogels aim to mimic the natural extracellular matrix (ECM) of tissues. They can be made of different materials, natural and synthetic, and many different hydrogels are commonly used in cell cultures (Ahmed, 2015). Because the ECM is different in different tissues there is need for hydrogels with

different properties, such as elasticity and stiffness. Different hydrogels are used with different cell types to create as *in vivo*-like tissue models as possible.

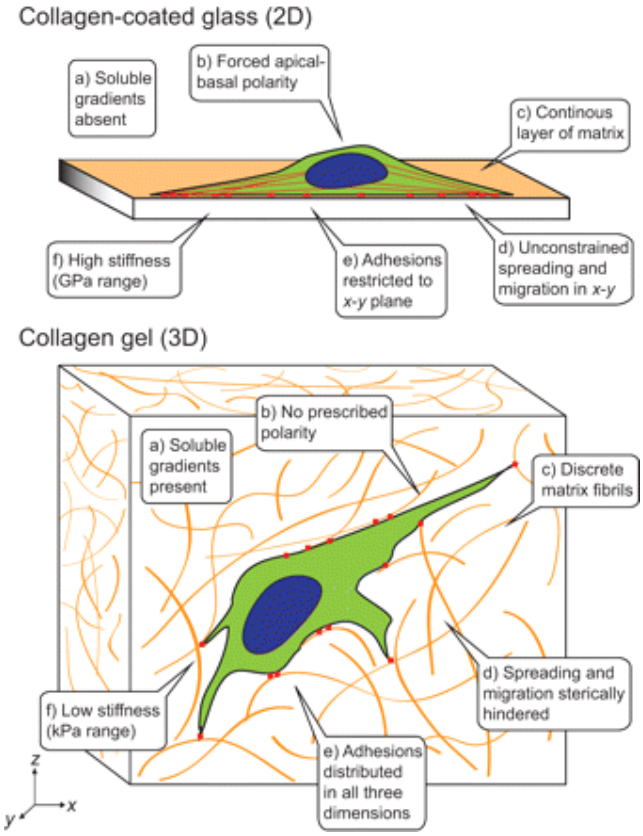


Figure 1. A schematic comparison of 2D and 3D cell cultures (Baker and Chen, 2012). In 2D cell culture cells are cultured on a flat surface, and the complexity of the cell cultures is lower than in 3D cultures. In 3D cultures, complex three-dimensional structures, such as blood vessels, can be established.

The hydrogels used in this thesis are collagen I and fibrin hydrogels. Also, a mixture of these two materials is used. The materials were selected based on previous studies conducted with neuronal and endothelial cell co-cultures (Osaki et al., 2018 and Campisi et al., 2018).

Type I collagen is the most abundant collagen in humans since it covers about 90% of all collagen in the body. Thus, it is a necessary structural ECM component in many tissues. These qualities make collagen I a good material for hydrogel studies. Usually type I collagen is derived from rat tail tendon, and it is commercially available as acid- or pepsin-solubilized type I collagen. (Caliari and Burdick, 2016 and Catoira et al., 2019) Collagen I hydrogel has been used in previous studies to establish 3D neuronal cell cultures (Honkamäki et al., 2021) as well as 3D vascular models (Liu et al., 2015) and 3D neuro-vascular co-cultures (Osaki et al., 2018).

Fibrin is formed naturally during wound coagulation. It is a polymer which is formed when thrombin, a serine protease, selectively cleaves fibrinogen, a dimeric glycoprotein. Formed fibrin molecules interact with each other through disulfide bonds. Fibrin is a widely used component in hydrogels

and it is commercially available derived from humans, which increases its biocompatibility properties. (Caliari and Burdick, 2016 and Catoira et al., 2019) Fibrin hydrogel has also been used in previous studies to establish 3D neuronal cell cultures (Bento et al., 2017), 3D vascular models (Liu et al., 2015) and 3D neuro-vascular co-cultures (Campisi et al., 2018).

1.2 Microfluidic devices for cell cultures

Conventional cell cultures performed on cell culture dishes, flasks and well-plates are still widely used and functional tools for various applications. However, it is hard to replicate the microenvironment and physiological conditions cells have *in vivo* in these cell culture platforms (Coluccio et al., 2019). With microfluidic cell culture devices, the goal is to create more *in vivo*–like microenvironment for the cells by controlling the physiological conditions, such as pH, flow rate and cell positioning (Park et al., 2015).

Microfluidic devices are essential for neuro-vascular co-culture studies for creating more *in vivo*-like tissue models. The formation of blood vessels can also be improved by creating flow in the cell cultures (Charbonier et al., 2018). Using conventional cell culture platforms is not optimal, because fluid flow cannot be created in these platforms and the microenvironment is not recapitulating the one found *in vivo*.

1.3 *In vitro* neuro-vascular models

Many of the existing 3D models in microfluidic devices focus on modeling CNS, more precisely the neurovascular unit (NVU), a functioning unit in the blood brain barrier (BBB) (Campisi et al., 2018, Nguyen et al., 2019, Lee et al., 2020). The NVU consists of neurons, perivascular astrocytes, microglia, pericytes, endothelial cells (EC), and the basement membrane (BM) (Bell et al., 2020). BBB acts as a semipermeable barrier between the circulating blood and brain and selects the substances that can pass through to CNS (Bhalerao et al., 2020). Therefore, the BBB protects CNS from unwanted substances and maintains the brain homeostasis. The existing studies have already provided more physiologically relevant BBB models to help in understanding the neurogenesis and angiogenesis in CNS and presented platforms for drug screening and disease modeling (Campisi et al., 2018, Nguyen et al., 2019, Lee et al., 2020).

BBB is a unique structure that requires realistic *in vitro* modeling of neuronal and vascular networks. There are also other models needed for modeling the innervation and vascularization of different kinds of tissues. There are not many existing studies focusing on modeling the neuro-vascular interactions found outside the BBB on microfluidic devices. A study by Osaki et al. established a 3D model of motor neurons and microvascular networks in a microfluidic device for studying the

paracrine interactions between the neurons and endothelial cells (Osaki et al., 2018). There is need for more accurate neuro-vascular models to study the interactions, developmental biology and drug screening possibilities between neuronal and vascular networks found in different tissues. And although there are many studies focusing on modeling the BBB or NVU, there is also a need for better models to capture the unique and complex structure of the BBB (Bhalerao et al., 2020). Establishing these improved models requires improvements in cell culture environment.

1.4 Aims of the study

In this thesis hydrogels are optimized for culturing human induced pluripotent stem cell (hiPSC) - derived neural progenitor cells and implementing the optimal conditions to microfluidic cell culture devices. The aim is to see how different cell culture media affect hydrogels and neuronal cell growth. The information from this thesis will be used for establishing 3D neuro-vascular co-culture studies.

The hydrogels used in the thesis are collagen I, fibrin and collagen I–fibrin hydrogels. The potential of these hydrogels for culturing the hiPSC-derived neural progenitor cells was researched to see how the cells and hydrogels act in different cell culture media. Used cell culture media are neural maturation medium (NMM), endothelial cell growth medium-2 (EGM-2) and 1:1 mixture medium of NMM and EGM-2. We examined the hydrogel integrity and cell growth in the studied conditions in 24-well-plate and microfluidic device.

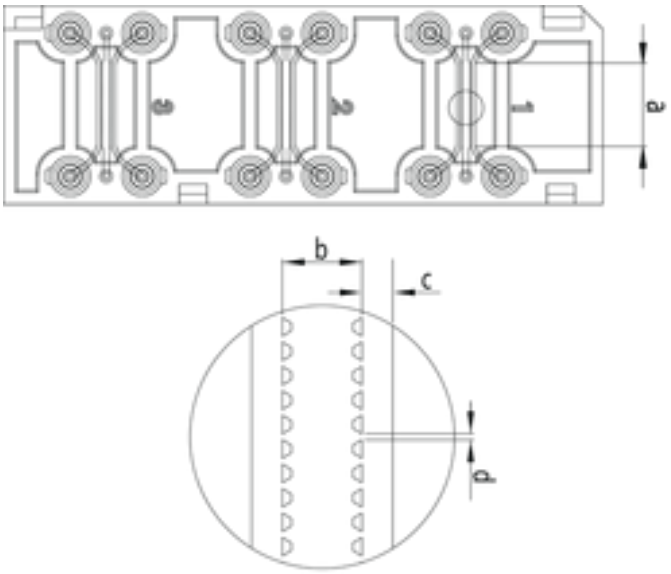
2. MATERIALS AND METHODS

2.1 Cells

The hiPSC-derived neural progenitor cells used in this study consist the derivatives of the in-house derived hiPSC line 04511.WTs (total passages 34, feeder-free passages 7). The cell line is derived at the Faculty of Medicine and Health Technology (MET), iPS Cells facility, Tampere University, Finland. The regional ethics committee of Pirkanmaa Hospital District has given supportive statements for the derivation, culture, and differentiation of hiPSCs (R08070). The differentiation of the in-house-produced neurons was performed according to the method in a previous publication (Hyvärinen et al., 2019).

2.2 Cell culture platforms

The platforms used for the cell cultures are a microfluidic chip and a 24-well-plate (Mattek, USA). The microfluidic chips used for the hydrogel optimization are 3D cell culture chips from AIM Biotech (3D Cell Culture Chips 3DT, AIM Biotech, Singapore). A schematic picture of the chip can be seen in Figure 2. Each chip has a 3-channel design, which means that each chip has 3 hydrogel channels. All three hydrogel channels are flanked by 2 media channels. Chips are made of polydimethylsiloxane (PDMS), which is permeable to gas. The addition of different volumes of medium to the upper and lower media channels creates interstitial flow of medium through the hydrogel channel (Figure 3). (AIM Biotech, viewed 18 January 2021, https://www.aimbiotech.com/chips.html)



Dimensions:

a: Length of channels	10.50 mm
b: Width of gel channel	1.30 mm
c: Width of media channels	0.50 mm
d: Gap between posts	0.10 mm
Height of channels	0.25 mm

Figure 2. The structure and the dimensions of the AIM Biotech 3D Cell Culture Chip. (AIM Biotech, viewed 18 January 2021, https://www.aimbiotech.com/chips.html)

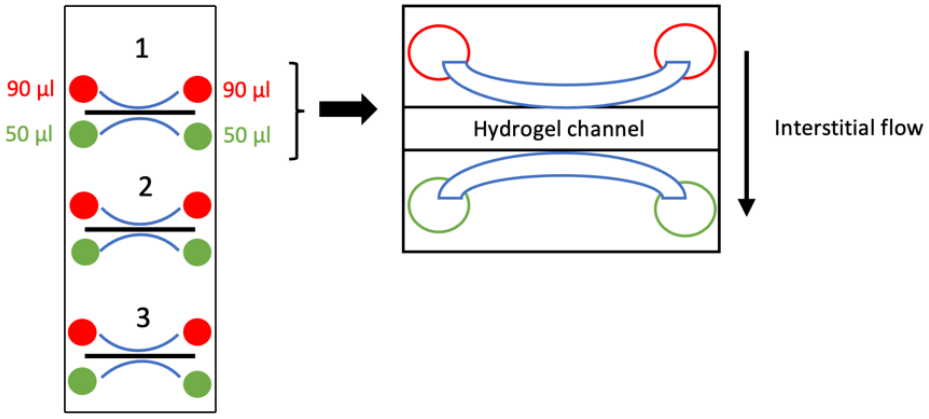


Figure 3. A schematic picture of the AIM Biotech 3D Cell Culture Chip structure showing how the interstitial flow is created. By adding different volumes of medium to the upper (red) and lower (green) medium reservoirs, hydrostatic pressure is created and the medium flows through the hydrogel channel creating interstitial flow. (AIM Biotech, viewed 16 April 2021, https://aimbiotech.com/product/3d-cell-culture-chips-3dt/)

2.3 Main reagents

2.3.1 Hydrogels

The hydrogels used for the cell cultures are fibrin hydrogel, collagen I hydrogel and collagen I–fibrin hydrogel. The materials were selected based on their biocompatibility properties and previous studies conducted with neuronal and endothelial cells. The fibrin hydrogel consists of 1:1 mixture of fibrinogen (Sigma-Aldrich, Germany) in Dulbecco's phosphate-buffered saline (DPBS) 5 mg/ml and thrombin (Sigma-Aldrich, Germany) in endothelial cell growth basal medium-2 (EBM-2, Lonza, Switzerland) 2 IU/ml. The cells are mixed with components of the hydrogel and incubated for approximately 30 min. During this time fibrinogen is converted to fibrin by thrombin mediated proteolysis.

The collagen I hydrogel consists of rat tail collagen I (Gibco, New Zealand) in DPBS 0.5 mg/ml, 1/10 10× phosphate buffered saline (PBS) and sterile H_2O. The collagen is neutralized with 1 M NaOH. The volume of 1 M NaOH usually varies and needs to be adjusted. The volume of 1 M NaOH is calculated as:

$$V_{stock\ collagen} \times 0.025$$

The cells are mixed with components of the hydrogel and incubated for approximately 30–40 min. During this time the hydrogel gelatinizes, and the gel is formed.

The collagen I–fibrin hydrogel consists of $^1/_3$ 3 mg/ml rat tail collagen I (Gibco, New Zealand) neutralized with 1 M NaOH, $^1/_3$ 2 IU/ml thrombin (Sigma-Aldrich, Germany) in EBM-2 (Lonza,

Switzerland) and $^1/_3$ 4.5 mg/ml fibrinogen (Sigma-Aldrich, Germany) in DPBS. The volume of 1M NaOH is calculated similarly as explained previously with collagen I hydrogel. As with fibrin and collagen I hydrogels, with collagen I–fibrin hydrogel the cells are mixed with components of the hydrogel and incubated for approximately 30–40 min. During this time the hydrogel gelatinizes, and the gel is formed.

2.3.2 Media

Media used for the 3D cell cultures are 1) EGM-2 (Lonza, Switzerland), 2) NMM (Hyvärinen et al., 2019) and 3) 1:1 mixture media of EGM-2 and NMM. EGM-2 is a commercial medium optimized for endothelial cells. Using EGM-2 in the future experiments with neuro-vascular co-cultures can be essential, and therefore it was used in the hydrogel optimization experiments. NMM has been optimized for neuronal cells and includes essential components for the neuronal cells. An ideal hydrogel for the co-culture experiments works in both media or in the mixture medium and thus provides beneficial microenvironment for all cell types.

NMM contains neural maintenance media (N3), which consists of 1:1 DMEM/F12 with GlutaMAX and Neurobasal, 0.5 % N2, 1 % B27 with retinoic acid, 0.5 mM GlutaMAX, 0.5 % NEA, 50 µM 2-mercaptoethanol, 2.5 µg/ml Insulin (Sigma-Aldrich, Germany) and 0.1 % penicillin/streptomycin (Lonza, Switzerland). If not mentioned, supplements are from Thermo Fischer Scientific (USA). N3 is supplemented with 20 ng/ml brain-derived neurotrophic factor (BDNF, R&D Systems, USA), 10 ng/ml glial-derived neurotrophic factor (GDNF, R&D Systems, USA), 500 µM dibutyryl-cyclicAMP (db-cAMP, Sigma-Aldrich, Germany) and 200 µM ascorbic acid (AA, Sigma-Aldrich, Germany) to create NMM. The supplements are added to N3 fresh before using the media. NMM containing 10 µM ROCK inhibitor (STEMCELL technologies, Canada) was used for the first day of culturing. Medium changes were performed every day for AIM Biotech chips, and every other to every third day for 24-well-plates.

2.4 Experimental procedure

The platforms for the cell cultures were microfluidic AIM Biotech chips and 24-well-plates. Hydrogel optimization was started with conventional 24-well plates with NMM medium. The information gained from the well plate experiments was then being utilized in the experiments performed in the AIM Biotech chip. The final optimization was aimed to be performed in the chip, since microfluidic devices with perfusion are essential for improving the formation of blood vessels and provide better microenvironment for the neuro-vascular co-culture studies.

2.4.1 Culturing cells in three hydrogels in 24-well-plate

Collagen I hydrogel was prepared by mixing 10× PBS, sterile H2O, 1M NaOH and collagen I solution for a total volume of 350 µl 0.5 mg/ml collagen I hydrogel. All the components, including the cells, were kept on ice to prevent unwanted gelling of the collagen I. The pH of the mixture was checked to be between 6.5 – 7.5 with pH-indicator strips. The hiPSC-derived neural progenitor cells were mixed and suspended in the collagen I hydrogel solution in the density of 10^6 cells/ml. 100 µl hydrogel/cell suspension was pipetted to one well of 24-well-plate. In total, 3 wells were plated with cells in collagen I hydrogel. The hydrogel was let to gelatinize in +37°C in an incubator for about 30 min before the addition of medium. 1000 µl NMM supplemented with 10 µM ROCK inhibitor was pipetted to each well.

Fibrin hydrogel was prepared by mixing and suspending the hiPSC-derived neural progenitor cells with 2 IU/ml thrombin in EBM-2 in the density of 10^6 cells/ml. Thrombin/cell suspension and 5 mg/ml fibrinogen in DPBS were mixed 1:1 for a total volume of 350 µl fibrin hydrogel. The fibrin hydrogel solution was plated to a 24-well-plate. 100 µl hydrogel/cell suspension was pipetted to one well of 24-well-plate. In total, 3 wells were plated with cells in fibrin hydrogel. The hydrogel was let to gelatinize in +37°C in an incubator for about 30 min before the addition of medium to the wells. Medium was added to fibrin hydrogel wells in a similar manner than the wells with collagen I hydrogel. 1000 µl NMM supplemented with 10 µM ROCK inhibitor was pipetted to each well.

Collagen I–fibrin hydrogel was prepared by mixing 1:1 3 mg/ml collagen I neutralized with 1 M NaOH and 2 IU/ml thrombin in EBM-2. The pH of the mixture was checked to be between 6.5 – 7.5 with pH-indicator strips. The IPSC-derived neural progenitor cells were suspended in 4.5 mg/ml fibrinogen in PBS in the density of 10^6 cells/ml. All the components, including cells, were kept on ice. Collagen I–thrombin mixture was mixed 2:1 with fibrinogen-cell mixture and mixed well. 100 µl hydrogel/cell suspension was pipetted to one well of 24-well-plate. In total, 3 wells were plated with cells in collagen I–fibrin hydrogel. The hydrogel was let to gelatinize in +37°C in an incubator for about 30 min before the addition of medium to the wells. Medium was added to fibrin hydrogel wells in a similar manner than the wells with collagen I- and fibrin hydrogels. 1000 µl NMM supplemented with 10 µM ROCK inhibitor is pipetted to each well.

The following day the medium is changed to NMM without ROCK inhibitor for all wells. Later the medium is changed every 2-3 days and the growth of the cells and the appearance of the hydrogel are being observed under a phase contrast microscope (Eclipse Ts2R, Nikon, Japan). The cell cultures are maintained for up to 14 days. Experiment was performed once. A schematic image of the 24- well-plate with different hydrogels can be seen in Figure 4.

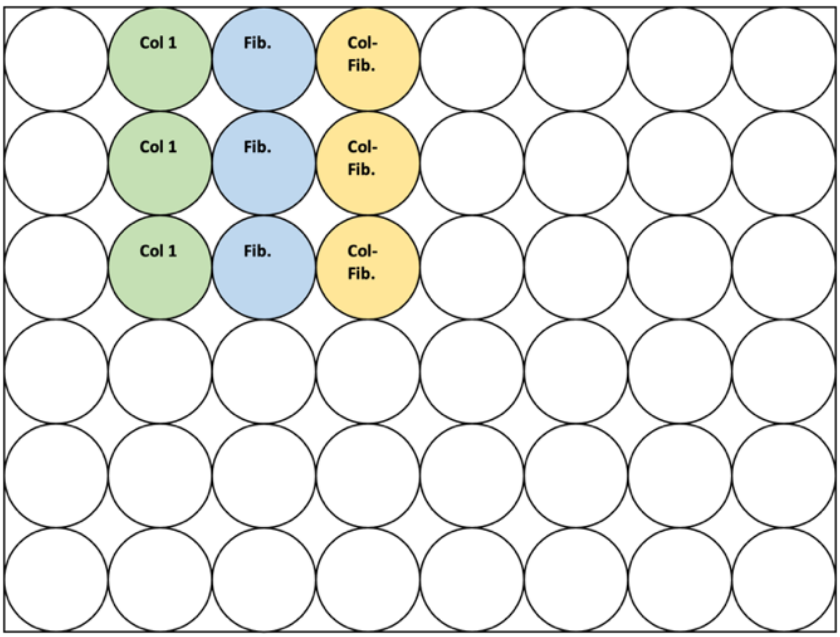


Figure 4. A schematic picture of the 24-well-plate plated with IPSC-derived neural progenitor cells in 3 different hydrogels. The used cell culture medium is NMM for all wells. Col 1 = Collagen I hydrogel (green), Fib. = Fibrin hydrogel (blue), Col-Fib. = Collagen I–fibrin hydrogel (yellow)

2.4.2 Culturing cells in two hydrogels in microfluidic chips

Collagen I hydrogel was prepared similarly than with 24-well-plate and the cell density remained the same 10^6 cells/ml with AIM Biotech chips. 10 µl hydrogel/cell suspension was pipetted to a hydrogel channel of an AIM Biotech chip. In total, 9 channels of 3 chips were plated with cells in collagen I hydrogel. The hydrogel was let to gelatinize in +37°C in an incubator for about 30 min before the addition of medium to the media channels. For each chip, different media was added. For chip 1. the medium was EGM-2, for chip 2. the medium was NMM supplemented with 10 µM ROCK inhibitor and for chip 3. the medium was 1:1 mixture medium of EGM-2 and NMM supplemented with 10 µM ROCK inhibitor (Figure 5.). The volumes of media were same for all chips. 15 µl medium was pipetted to all media channels of the chip. In addition, 90 µl medium was pipetted to the upper media reservoirs and 50 µl medium was pipetted to the lower media reservoirs to create the interstitial flow through the hydrogel channel. The media volumes for chips are demonstrated in Figure 5.

Collagen I–fibrin hydrogel was prepared similarly than with 24-well-plate and the cell density remained the same. For each hydrogel channel of the AIM Biotech chip, 30 µl hydrogel was prepared and 10 µl was pipetted into the hydrogel channel. In total, 9 channels of 3 chips were plated with cells in collagen I–fibrin hydrogel. The hydrogel was let to gelatinize in +37°C in an incubator for about 30 min before the addition of medium to the media channels. Three different media were used also for collagen I–fibrin hydrogels. Media was added to collagen I–fibrin hydrogel chips in a similar manner than the chip with collagen I hydrogel. The media volumes for chips with collagen I–fibrin hydrogel and collagen I are demonstrated in Figure 5. Experiment with AIM Biotech chips was performed once.

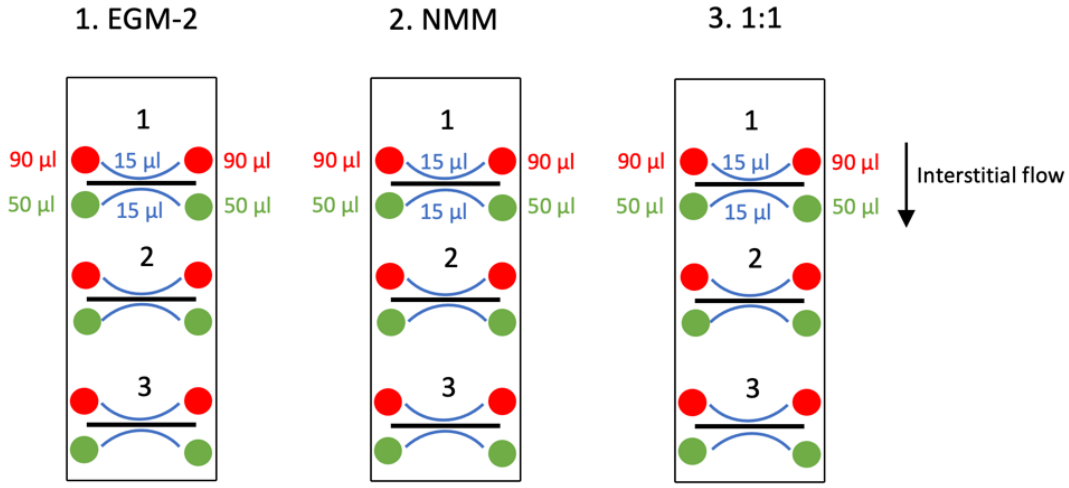


Figure 5. A schematic image of the media volumes added to the AIM Biotech chips. First, 15 µl medium is added to the medium channels (blue). Second, 90 µl medium is added to the upper medium reservoirs (red) and 50 µl medium is added to the lower medium reservoir (green) to create the interstitial flow. Used medium: Chip 1. = EGM-2, Chip 2. = NMM, Chip 3. = 1:1 mixture of EGM-2 and NMM.

The following day NMM without ROCK inhibitor is used for the chip 2. that was plated with NMM supplemented with 10 µM ROCK inhibitor and for chip 3. that was plated with 1:1 mixture medium of EGM-2 and NMM supplemented with 10 µM ROCK inhibitor. Later the media are changed every day and the growth of the cells and the appearance of the hydrogel are being observed under a phase contrast microscope. The cell cultures are maintained for up to 14 days.

3. RESULTS

3.1 Hydrogels in cell culture

3.1.1 Collagen I hydrogel in 24-well-plate and microfluidic chip

Phase contrast images of the collagen I hydrogels on day 13 in AIM Biotech chips in different cell culture media can be seen in Figure 6. Collagen I hydrogels stayed intact in 24-well-plate in NMM for 14 days. No degradation or shrinkage was detected during the culturing period when observing the samples under a phase contrast microscope. The collagen I hydrogels in AIM Biotech chip in NMM also stayed intact for 14 days, and similarly as in 24-well-plate, no degradation or shrinkage was detected (Figure 6A). In AIM Biotech chip, no cells had sprouted outside the hydrogel to the media channels. The cultures were observed under a phase contrast microscope and the growth of the neuronal cells was normal in both 24-well-plate and microfluidic chip.

Collagen I hydrogels in the 1:1 mixture media of EGM-2 and NMM in the AIM Biotech chip started to shrink during the 14-day culturing period (Figure 6B). The shrinkage started after 7 days. When observed under a phase contrast microscope, more cell aggregates were detected in collagen I hydrogel in 1:1 medium compared to collagen I hydrogel in NMM (Figure 6A) The aggregates started to form after 7 days of culturing. The aggregates and the shrinkage of the hydrogel can be seen in Figure 6. In Figure 7, samples are imaged on day 6, and no major aggregates nor gel shrinkage between the pillars of the chip can be seen in NMM or 1:1 mixture medium (Figure 7A&B). Some cells had sprouted outside the hydrogel and adhered to the bottom of the media channels during the 14-day culturing period in 1:1 mixture medium. The growth of the adherent cells in the media channels looked different compared to the cells in the hydrogel when observed under a phase contrast microscope.

Collagen I hydrogels in EGM-2 in microfluidic chip started to shrink more compared to the samples in NMM and 1:1 mixture media during the 14-day culturing period. The shrinkage can be observed in Figure 6C and Figure 7C. In Figure 7, the 10X magnification images show how the hydrogels in NMM and 1:1 mixture medium (A&B) have not shrunk between the pillars of AIM Biotech chips on day 6, but in EGM-2 (C) the hydrogel has already started to shrink. In Figure 6, the shrinkage can be observed on day 13, and the major shrinkage can be observed in Figure 6C, collagen I hydrogel in EGM-2. Similarly, as in 1:1 mixture media, in EGM-2 cells with divergent growth sprout outside

the hydrogel and adhered to the bottom of the media channels. Compared to the samples in 1:1 medium, there were more adherent cells on the bottom of the media channels in EGM-2 medium.

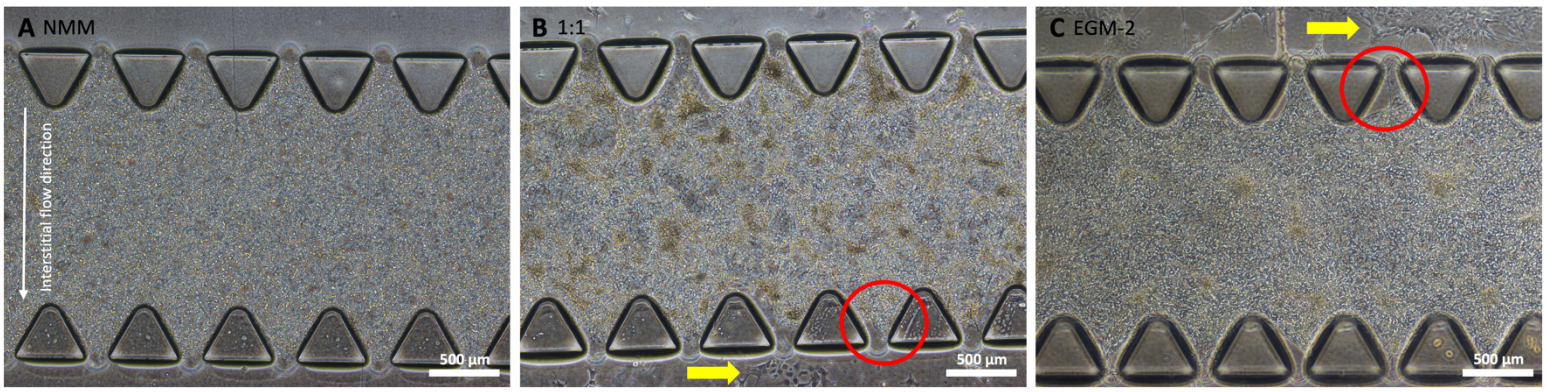


Figure 6. Phase contrast images of hIPSC-derived neural progenitor cells in collagen I hydrogel in 3 different media taken with 4× magnification on day 13. The red circles show part of the shrinkage of the hydrogel and the yellow arrows point the cell growth in the media channels. A: NMM, B: 1:1 mixture medium of NMM and EGM-2, C: EGM-2.

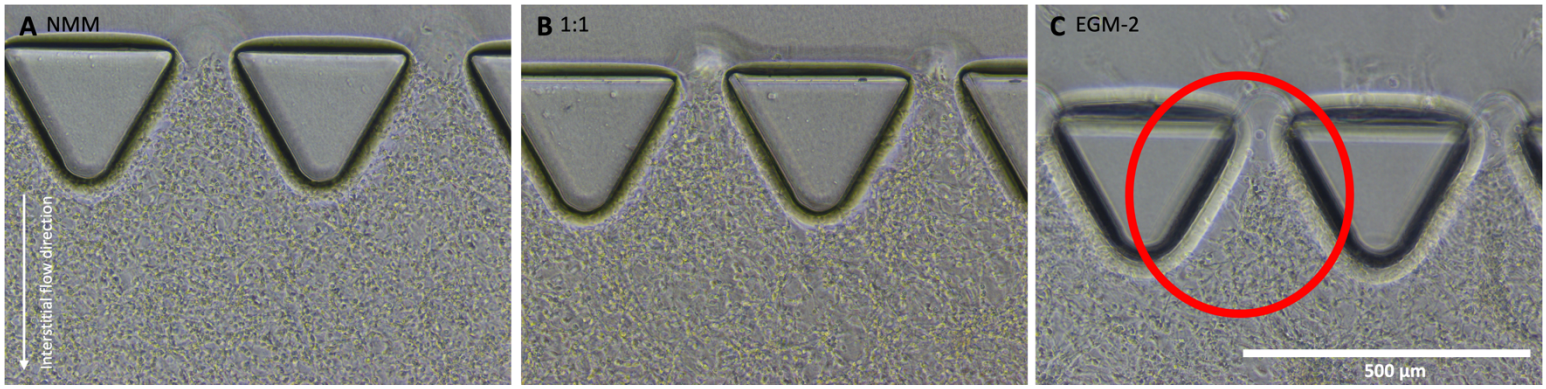


Figure 7. Phase contrast images of hIPSC-derived neural progenitor cells in collagen I hydrogel in 3 different media taken with 10× magnification on day 6. The red circle shows the shrinkage in the hydrogel in EGM-2 medium. A: NMM, B: 1:1 mixture medium of NMM and EGM-2, C: EGM-2.

3.1.2 Fibrin hydrogel in 24-well-plate

The fibrin hydrogels in NMM in 24-well-plate started to degrade rapidly during the first day of culturing. By the day 2 the gel had degraded almost completely. A phase contrast image of fibrin hydrogels in 24-well-plate on day 2 can be seen in Figure 8. Because of the rapid degradation of the fibrin hydrogel in 24-well-plate, no experiments were performed with fibrin hydrogels in AIM Biotech chips.

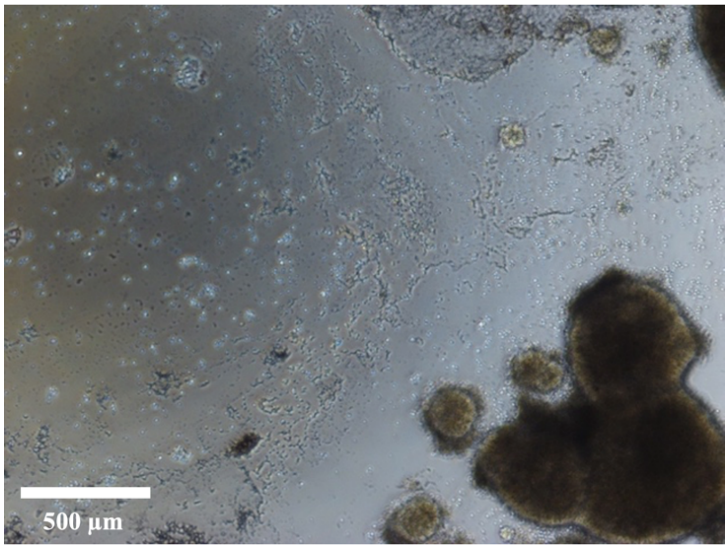


Figure 8. Phase contrast image of hIPSC-derived neural progenitor cells in fibrin hydrogel in NMM in 24-well plate taken with 4× magnification on day 2.

3.1.3 Collagen I–fibrin hydrogel in 24-well-plate and microfluidic chip

Collagen I–fibrin hydrogels in 24-well-plate in NMM started to shrink and detach from the bottom of the well plate during the 14-day culturing period. The growth of the cells stayed normal despite the degradation when observed under a phase contrast microscope. The collagen I–fibrin hydrogels in the AIM Biotech chip also started to shrink and degrade in the chip, more rapidly than in the 24-well-plate. The shrinkage happened in all 3 cell culture media. This rapid shrinkage in AIM Biotech chip can be seen in images taken on day 3 in Figure 9. There was no significant difference between the samples in NMM (Figure 9A), 1:1 mixture medium (Figure 9B) and EGM-2 (Figure 9C).

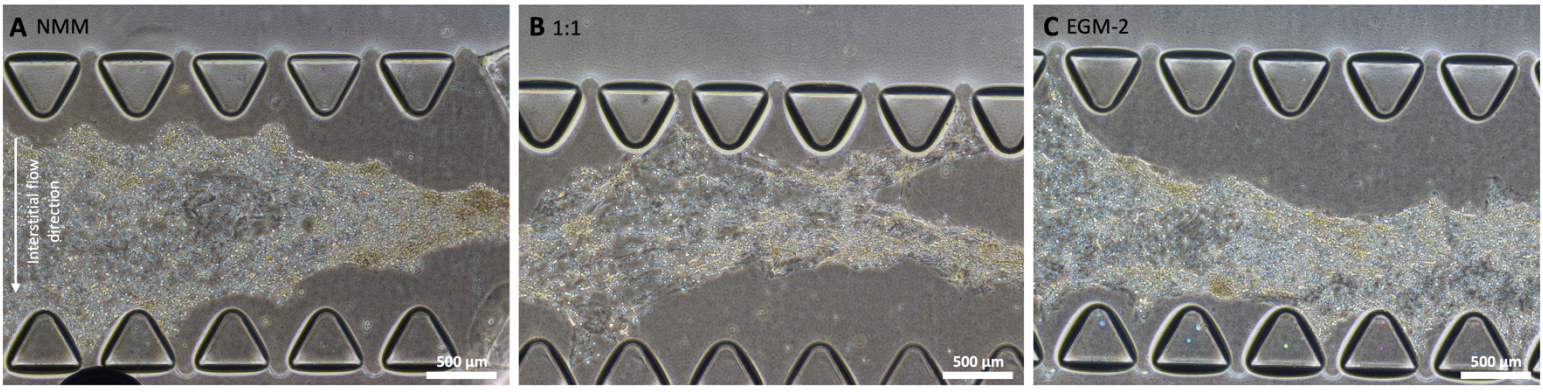


Figure 9. Phase contrast images of hIPSC-derived neural progenitor cells in 3 different cell culture media in collagen I–fibrin hydrogel in AIM Biotech chip taken with 4× magnification on day 3. A: EGM-2, B: NMM, C: 1:1 mixture medium of NMM and EGM-2.

4. DISCUSSION

In the study, culturing hiPSC-derived neural progenitor cells was tested in three hydrogels and three cell culture media. All used hydrogels, collagen I, fibrin and collagen I–fibrin, were tested in 24-well-plate in NMM. Collagen I and collagen I–fibrin hydrogels were tested also in AIM Biotech chips in three different cell culture media including NMM, EGM-2 and 1:1 mixture media of NMM and EGM-2. The cell cultures were maintained for up to 14 days. The growth of the cells and the appearance of the hydrogel were being observed under a phase contrast microscope during the culturing period.

The fibrin hydrogel cultures in 24-well-plate in NMM degraded rapidly during the first days of culturing. Because of the strong degradation, the tests with fibrin hydrogels were not continued in the AIM Biotech chips. The strong degradation can be caused by the cells, because fibrin hydrogels are extremely sensitive to protease mediated fibrinolysis (Caliari and Burdick, 2016). There are many enzymes degrading fibrin, for example plasmin and matrix metalloproteinases (MMPs), which are produced by almost all cell types (Lorentz et al., 2011). In the study by Bang et al. a neurovascular BBB model was established in fibrin hydrogel in microfluidic platform. The study does not report the degradation of fibrin during the 7-day culturing period. However, aprotinin, a serine-protease inhibitor, is used in the study. (Bang et al., 2017) This protease inhibitor prevents the plasmin-mediated degradation, which can be the reason why fibrin hydrogel stays intact in the study (Ahmed et al., 2007). Aprotinin was also used in the study by Bento et al. to establish 3D neuronal network in fibrin hydrogel (Bento et al., 2017). Therefore, the use of aprotinin could enable the use of fibrin hydrogel in neuro-vascular co-culture studies. The hydrogel should also be tested in microfluidic platform to confirm the suitability in different applications.

The collagen I–fibrin hydrogel cultures in 24-well-plate in NMM started to shrink during the 14-day culturing period. When culturing in AIM Biotech chips in different media, the hydrogels shrunk more than the samples in 24-well-plate. Hydrogels shrunk in chips in all cell culture media, without noticeable difference between the samples in different media when observed under a phase contrast microscope. The differences in shrinkage between samples in 24-well-plate and AIM Biotech chips can be due to the flow in the chips. Interstitial flow is created to the chips by adding different volumes of media to the media reservoirs and thus by creating hydrostatic pressure. This flow creates physiological forces that can help the hydrogel to detach from the bottom of the chip and contribute to the shrinking process. In 24-well-plate, such physiological forces are not present. However, the growth of the neuronal cells seemed normal on the 24-well-plate when observed under a phase contrast microscope. The shrinkage can be due to the strong degradation of fibrin that was detected

previously with fibrin hydrogel. The collagen I–fibrin hydrogel can possibly be used in future applications if the degradation of fibrin can be prevented. This can be achieved for example, by using the aforementioned protease inhibitor aprotinin or another similarly functioning inhibitor ε-aminocaproic acid (ACA), to prevent the cells from degrading fibrin (Lorentz et al., 2011 and Catoira et al., 2019). In the study by Schuh et al. Schwann cells were seeded inside collagen I–fibrin hydrogel and an optimal blend of collagen I and fibrin was provided to support neurite out growth. Again, aprotinin was used to prevent the degradation of fibrin. A blend of 90% collagen I and 10% fibrin was found to be optimal for the cells. (Schuh et al., 2018) The use of aprotinin and modifications in the collagen-fibrin ratio can be tested in the future to improve the collagen I–fibrin hydrogel and potentially use it in the neuro-vascular co-cultures.

In the study, collagen I hydrogel was found to be useful when culturing hiPSC-derived neural progenitor cells in NMM. However, the study showed differences in collagen I hydrogel cultures in different cell culture media. In NMM, cultures in both 24-well-plate and AIM Biotech chip stayed intact for the 14-day culturing period. Also, the growth of the cells seemed typical for neurons when observed under a phase contrast microscope. There were no cells growing outside the hydrogel region. In EGM-2 the hydrogels started to shrink during the 14 days in AIM Biotech chip. There were also cells sprouting out from the hydrogel to the bottom of the media channels. The growth of the cells was different compared to the neuronal cells in NMM. EGM-2 is commercial medium for endothelial cells, and it contains many angiogenic growth factors eg. vascular endothelial growth factor (VEGF) (Lonza, viewed 24 February 2021, https://bioscience.lonza.com/lonza_bs/CH/en/Primary-and-Stem-Cells/p/000000000000185303/EGM-2-Endothelial-Cell-Growth-Medium-2-BulletKit). In the study by Bang et al. the effect of EGM-2 to neuronal cells was studied. The study reports that in neurons cultured in EGM-2 the synaptic connectivity is decreased compared to the neurons cultured in neuronal medium. The study also reports that VEGF in EGM-2 has pro-proliferative and pro-migratory effects in neurons. (Bang et al., 2017) These results support the untypical cell growth to the media channels that was detected with neuronal cells in EGM-2. More detailed characterizations are needed to determine the morphology of the cells in the media channel. The samples in 1:1 mixture medium of EGM-2 and NMM showed similarities to the samples in EGM-2. In 1:1 mixture media, cells also started to sprout out from the hydrogel. However, less cells were sprouting compared to the samples in EGM-2. Samples in mixture medium also started to shrink during the culturing period, but the shrinkage started later and was minor compared to the samples in EGM-2. It seems that using EGM-2 causes the shrinkage and affects the cells, which then start to sprout out from the hydrogel. EGM-2 might not itself cause the hydrogel shrinkage, but since it affects the cell behavior, it can induce the cells into shrinking the hydrogel. The effects of EGM-2 are milder in the samples in the 1:1 mixture media, in which the concentration of EGM-2 is smaller. In 1:1 mixture medium the cells started to aggregate more

during the culturing period than in the EGM-2 or NMM. This can possibly be caused by the mixture medium, but more tests need to be performed to define the mechanism.

Collagen I hydrogel was the only hydrogel tested which stayed intact for 14 days in the AIM Biotech chip. This was achieved only when culturing the cells in NMM. Because EGM-2 medium is commonly used for endothelial cells, it might be essential in the neuro-vascular co-culture studies. The collagen I hydrogel might not be the best option since it reacts strongly to EGM-2. As discussed earlier, testing protease inhibitors, such as aprotinin, to collagen I–fibrin and fibrin hydrogels is needed to examine the possibility to prevent the cells from degrading fibrin. More studies need to be performed to optimize hydrogel for neuro-vascular co-cultures, because the used cell type and cell culture medium affect the growth of the cells and the integrity of the hydrogel.

5. CONCLUSIONS

The hydrogel optimization performed in this study serve as a basis for finding optimal hydrogel for 3D neuro-vascular co-cultures performed in microfluidic cell culture device. hiPSC-derived neural progenitor cells were cultured in collagen I, fibrin and collagen I–fibrin hydrogels. Fibrin hydrogel was found to degrade rapidly in cell cultures. Three different cell culture media were tested for collagen I and collagen I–fibrin hydrogels in AIM Biotech chips. Collagen I hydrogel stayed intact during the culturing period in one cell culture media, while collagen I–fibrin hydrogels degraded and shrunk. Used media affected the collagen I hydrogel integrity. More studies need to be performed to optimize hydrogel and cell culture media for neuro-vascular co-cultures.

REFERENCES

AGER, A., 2017. High Endothelial Venules and Other Blood Vessels: Critical Regulators of Lymphoid Organ Development and Function. *Frontiers in Immunology,* **8**, pp. 45.

AHMED, E.M., 2015. Hydrogel: Preparation, characterization, and applications: A review. *Journal of Advanced Research,* **6**(2), pp. 105–121.

AHMED, T.A.E., GRIFFITH, M. and HINCKE, M., 2007. Characterization and Inhibition of Fibrin Hydrogel–Degrading Enzymes During Development of Tissue Engineering Scaffolds. *Tissue engineering,* **13**(7), pp. 1469–1477.

BAKER, B.M. and CHEN, C.S., 2012. Deconstructing the third dimension – how 3D culture microenvironments alter cellular cues. *Journal of cell science,* **125**(13), pp. 3015.

BANG, S., LEE, S., KO, J. et al., 2017. A Low Permeability Microfluidic Blood-Brain Barrier Platform with Direct Contact between Perfusable Vascular Network and Astrocytes. *Scientific Reports,* **7**(1), pp. 8083.

BELL, A.H., MILLER, S.L., CASTILLO-MELENDEZ, M. et al., 2020. The Neurovascular Unit: Effects of Brain Insults During the Perinatal Period. *Frontiers in Neuroscience,* **13**, pp. 1452.

BENTO, A.R., QUELHAS, P., OLIVEIRA, M.J. et al., 2017. Three-dimensional culture of single embryonic stem-derived neural/stem progenitor cells in fibrin hydrogels: neuronal network formation and matrix remodelling. *Journal of Tissue Engineering and Regenerative Medicine,* **11**(12), pp. 3494–3507.

BHALERAO, A., SIVANDZADE, F., ARCHIE, S.R. et al., 2020. In vitro modeling of the neurovascular unit: advances in the field. *Fluids and Barriers of the CNS,* **17**(1), pp. 22.

CHARBONIER, F.W., ZAMANI, M. and HUANG, N.F., 2018. Endothelial Cell Mechanotransduction in the Dynamic Vascular Environment. Advanced Biosystems, 3(2), pp. 1800252.

CALIARI, S.R. and BURDICK, J.A., 2016. A practical guide to hydrogels for cell culture. *Nature Methods,* **13**(5), pp. 405–414.

CAMPISI, M., SHIN, Y., OSAKI, T. et al., 2018. 3D self-organized microvascular model of the human blood-brain barrier with endothelial cells, pericytes and astrocytes. *Biomaterials,* **180**, pp. 117–129.

CATOIRA, M.C., FUSARO, L., DI FRANCESCO, D. et al., 2019. Overview of natural hydrogels for regenerative medicine applications. *Journal of Materials Science: Materials in Medicine,* **30**(10), pp. 115.

COLUCCIO, M.L., PEROZZIELLO, G., MALARA, N. et al., 2019. Microfluidic platforms for cell cultures and investigations. *Microelectronic Engineering,* **208**, pp. 14–28.

GODDARD, L.M. and IRUELA-ARISPE, M., 2013. Cellular and molecular regulation of vascular permeability. *Thrombosis and haemostasis,* **109**(3), pp. 407–415.

HONKAMÄKI, L., JOKI, T., GRIGORYEV, N.A. et al., 2021. Novel method to produce a layered 3D scaffold for human pluripotent stem cell-derived neuronal cells. *Journal of neuroscience methods,* **350**, pp. 109043.

HYVÄRINEN, T., HYYSALO, A., KAPUCU, F.E. et al., 2019. Functional characterization of human pluripotent stem cell-derived cortical networks differentiated on laminin-521 substrate: comparison to rat cortical cultures. *Scientific Reports,* **9**(1), pp. 17125.

LEE, S., CHUNG, M., LEE, S. et al., 2020. 3D brain angiogenesis model to reconstitute functional human blood–brain barrier in vitro. *Biotechnology and bioengineering,* **117**(3), pp. 748–762.

LIU, J., ZHENG, H., POH, P.S.P. et al., 2015. Hydrogels for Engineering of Perfusable Vascular Networks*. International Journal of Molecular Sciences,* **16**(7), pp. 15997–16016.

LORENTZ, K.M., KONTOS, S., FREY, P. et al., 2011. Engineered aprotinin for improved stability of fibrin biomaterials. *Biomaterials,* **32**(2), pp. 430–438.

NGUYEN, E.H., DOMBROE, M.J., FISK, D.L. et al., 2019. Neurovascular Organotypic Culture Models Using Induced Pluripotent Stem Cells to Assess Adverse Chemical Exposure Outcomes. *Applied In Vitro Toxicology*, **5**(2), pp. 92–110.

OSAKI, T., SIVATHANU, V. and KAMM, R.D., 2018. Engineered 3D vascular and neuronal networks in a microfluidic platform. *Scientific Reports,* **8**(1), pp. 5168.

PARK, D., LIM, J., PARK, J.Y. et al., 2015. Concise Review: Stem Cell Microenvironment on a Chip: Current Technologies for Tissue Engineering and Stem Cell Biology. *STEM CELLS Translational Medicine,* **4**(11), pp. 1352–1368.

SCHUH, C. M. A. P., DAY, A.G.E., REDL, H. et al., 2018. An Optimized Collagen-Fibrin Blend Engineered Neural Tissue Promotes Peripheral Nerve Repair. *Tissue Engineering Part A,* **24**(17–18), pp. 1332–1340.

STILES, J. and JERNIGAN, T.L., 2010. The Basics of Brain Development. *Neuropsychology review,* **20**(4), pp. 327–348.

ZHANG, Y.S. and KHADEMHOSSEINI, A., 2017. Advances in engineering hydrogels. *Science,* **356**(6337), pp. Eaaf3627.