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Hyaluronic Acid Hydrogel as a Scaffold for Cells' Encapsulation

Susanna Wärmegård

Civilingenjörsprogrammet i molekylär bioteknik

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Abstract

Hydrogels are high water-content polymers that mimic the extracellular matrix of cells. The polymers can have many sources and be of natural origin from the extracellular matrix (ECM) of cells or be synthetically derived. Two such polymers are hyaluronic acid and gelatin, which can with the help of the release of free radicals from photoinitiators, initiated by UV light, polymerise, and form a hydrogel. In these hydrogels, cells can be encapsulated. The hydrogels can in turn be used to maintain cells as they are in the natural environment. For example, hydrogels can provide an in-vivo-like ECM for stem cells and endothelial cells by supporting "stemness" and cell-to-cell contact; respectively. We aim to establish a protocol for culturing cells in the hydrogel as a first milestone in a project focused on profiling the metabolome of cells grown in hydrogels. To accomplish this, four types of cells, namely mouse brain microvascular endothelial cells (bEnd.3), human umbilical vein endothelial cells (HUVECs), adult human lung fibroblast (hLFs) and mesenchymal stem cells (MSCs), were evaluated for growth in hyaluronic acid methacrylate (HA-ma), hyaluronic acid acrylamide (HA-am) as well as a QuattroGel composed by gelatin methacryloyl (GelMA), HA-ma, fibrinogen and thrombin. It was found that HA-ma supported viability and the stemness of mesenchymal stem cells, of which the metabolome can be further studied in order to evaluate the difference between regular 2D maintenance and maintenance in 3D. No sprouting was observed for the other cells encapsulated in the hydrogel, and further experiments are needed to find the source of error.

Teknisk-naturvetenskapliga fakulteten

Uppsala universitet, Utgivningsort Uppsala

Handledare: Samah Aboushariehа Ämnesgranskare: Morteza Aramesh

Examinator: Johan Åqvist

Till mormor

Populärvetenskaplig sammanfattning

Nu i examenstider kanske du äter en tårta, och kanske till och med en frukttårta med frukter ovanpå som är täckta av gelé. Detta gelélager är ofta gelatin, vilket är en polymer, alltså en lång kedja av molekyler. Förutom en vanlig ingrediens i tårta, finns det också mycket gelatin i kroppen. På samma sätt som gelatin på en tårta omringar jordgubbarna och vindruvorna, omringar gelatin de olika typerna av celler i kroppen, i något som kallas den extracellulära matrixen. En liknande polymer är hyaluronsyra, som finns i vissa fuktbevarande krämer och är ett av de vanligaste ämnena i den extracellulära matrixen mellan cellerna i kroppen. Dessa två material kan därför vara en bra modell för att undersöka hur celler beter sig i kroppen, men i ett system som går att undersöka i ett labb. När dessa polymerer bildar en gel kallas det för en hydrogel, då de innehåller mycket vatten. För att en hydrogel ska bildas, kan ultraviolett ljus och en speciell kemikalie användas, som reagerar och kopplar ihop de långa kedjorna. Eftersom både gelatin och hyaluronsyra är vanliga ämnen i kroppen, är det lätt att förstå att cellerna borde tycka om en miljö som dom utgör. Genom att tillsätta protein i blandningen kan hydrogelerna anpassas för cellerna, och få lämpliga egenskaper. Tack vare att polymererna kan hålla så mycket vatten, kan ämnen som cellerna behöver lätt transporteras genom hydrogelen.

Hydrogeler kan ha många applikationer som odling av celler för att undersöka hur de fungerar i kroppen, fast i ett system utanför kroppen. Med ett lyckat system, kan det i förlängningen användas till att utsätta en hydrogel med celler i för ett läkemedel, för att se hur cellerna reagerar på det. Det skulle potentiellt kunna minska behovet av djurförsök, och genom att kunna testa läkemedel på mänskliga celler skulle det ge bättre data på hur det faktisk kommer fungera i människokroppen. Ett annat område hydrogeler kan användas till är som ett stöd för celler att reparera skadad vävnad i kroppen. Hydrogelen skulle då kunna hjälpa kroppen att hela sig själv, eller genom att tillsätta celler i hydrogelen som bygger upp en ny vävnad.

I det här projektet har olika typer av celler blandats med dessa polymerer och bildat en hydrogel, för att ge möjlighet till cellerna att växa som de skulle gjort i kroppen. Hyaluronsyra har dels använts för sig i en hydrogel, dels tillsammans med gelatin. Cellerna som har använts är celler som kommer från blodkärl i en mushjärna eller blodkärl från en mänsklig navelsträng. Eftersom de kommer från blodkärl var ett mål med projektet att se avlånga celler i stället för runda celler i hydrogelen. Det upptäcktes att cellerna inte kunde bli avlånga i de prövade hydrogelerna, trots att flera olika varianter testades. I detta projekt hittades ingen orsak till att cellerna fortsatte vara runda, därför krävs fortsatta studier for att hitta anledningen. En annan typ av celler som testades var stamceller, där målet var att behålla stamcellerna runda i hydrogelen, i motsats till de andra testade cellerna. Detta för att de ska kunna anta vilken form som helst i ett senare skede. Vi lyckades visa att stamcellerna kunde behållas runda i en typ av hydrogel, vilket banar väg för framtida studier i hur cellerna mår när de får växa i hydrogel.

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
BBB	Blood-brain barrier
bFGF	Basic fibroblast growth factor
bEnd.3	Mouse brain microvascular endothelial cell
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix
FBS	Fetal bovine serum
GelMA	Gelatin methacryloyl
HA	Hyaluronic acid
HA-am	Hyaluronic acid acrylamide
HA-ma	Hyaluronic acid methacrylate
HG	High glucose
hASCs	Human adipose-derived stem cells
hLFs	Adult human lung fibroblast
HUVECs	Human umbilical vein endothelial cells
mM	Millimolar
MSCs	Mesenchymal stem cells
LAP	Lithium phenyl-2,4,6-trimethylbenzoylphosphinate
LG	Low glucose
PBS	Phosphate buffer saline
PDMS	Polydimethylsiloxane
RGD	Arginine-glycine-aspartate
TEA	triethylamine
UV	Ultra violet
VEGF	Vascular endothelial growth factor

1 Introduction

Imagine a future in which pharmaceuticals are developed without a single human or animal being put at risk, and the possibility to investigate the human body outside the body. How would that be possible, you might ask yourself. One approach is to use 3D hydrogels for cell cultivation, which opens the possibility to study how for example a pharmaceutical affect human cells, without exposing an actual human, or study how cells behave in a body-like environment. Even though hydrogels have been around for a couple of years, there are still many applications to explore and optimisation to do. Evaluation for each new type of cell is needed, to see if growth even is feasible. This project aims to assess the growth of different kinds of cells in different kinds of hydrogels.

1.1 Cell microenvironment

Cells *in vivo* are surrounded by extracellular matrix, ECM, which contains important proteins that provide binding sites for cells and structure for the cells to proliferate (Barthes *et al.* 2014). The cell microenvironment needs to contain everything the cells needs for the sake of survival, which traditionally is done *in vitro* in a flask with media with all sufficient nutrients. Though, this is a 2D environment, where the cells only can go in two directions, in contrast to the native environment where the cell can go in three directions. For this, a scaffold that support cell proliferation in three dimensions as well as the sufficient delivery of media is needed. Hydrogels with its unique properties can be such a scaffold, which will be discussed in the following sections.

1.2 Hydrogels

Hydrogels are gel materials that consist of crosslinked hydrophilic polymers, which can be either biological or synthetic in origin (Peppas *et al.* 2006). Some examples of polymers that can be used to form hydrogels include collagen, gelatin, and hyaluronic acid. Hydrogels mimic the *in vivo* extracellular matrix (ECM) with their high-water content and high permeability for oxygen and nutrients. Due to these mechanical and physical properties, hydrogels have numerous applications, and are therefore considered promising candidates for biomimetic cell-culture scaffolds. They can be used for cell culturing, regenerative medicine and to form scaffolds for cells (Melchels *et al.* 2012). The polymer can, together with a photoinitiator and UV light crosslink and form a hydrogel in which cells can be incorporated and cultivated. In contrast to regular 2D models, in which cells are maintained in flasks with media, 3D scaffolds more closely mimic the natural extracellular environment, resulting in cells with more realistic cellular responses (Kapałczyńska *et al.* 2018). This gives the possibility to study how cells behave *in vivo* in an *in vitro* environment, which provides data that are more representative of the natural environment. In order to conduct a study using hydrogels, a number of factors need to be considered. First, a suitable polymer for the

application needs to be selected, as well as the crosslinking mechanism. These parameters will be explained and discussed in more details in the following sections.

1.2.1 Hyaluronic acid hydrogel

Hyaluronic acid, HA, is a glycosaminoglycan that is present in the extracellular matrix, ECM, of cartilage and tendons (Nelson DL & Cox MM 2005) as well as in the brain (Zimmermann & Dours-Zimmermann 2008). It is built up by alternating D-glucuronic acid and *N*-acetylglycosamine, which in the body can be up to 50 000 repeats (Nelson DL & Cox MM 2005). Hydrogels based on HA are considered to be natural since they can be derived from biological sources (Peppas *et al.* 2006), although it can also be synthetically derived, and the backbone of HA can be functionalized in different ways. Porras Hernández *et al.* (2020) tested arginine-glycine-aspartate-(RGD)-functionalized hyaluronic acid acrylamide (HA-am) hydrogel for the proliferation and adhesion of mouse brain microvascular endothelial cells, bEnd.3 cell line, in 2D. The cells were proven to adhere in 2D, where the Young's modulus was between 6.32-10.20 kPa, and the hydrogels were crosslinked with UV. The Young's modulus is the elastic properties within a material, where a higher number means a stiffer material. The brain has a Young's modulus of around 1 kPa while bone have a Young's modulus of 20 GPa. HA does not promote any cell adhesion sites (Tenje *et al.* 2020), so an adhesion protein is needed for the cells to sprout. The adhesion peptide RGD is the minimal recognition sequence of many integrins, which can bind the cell to the ECM (Humphries *et al.* 2006). Adhesion peptides, such as RGD, needs to be incorporated with the polymer to provide binding sites for the cells, and in previous work, RGD has been incorporated with the help of either UV light exposure (Porras Hernández *et al.* 2020) or overnight incubation (Khetan & Corey 2019). In this project, RGD was incorporated in the hydrogel to promote cell binding sites in the 3D environment.

1.2.2 Methacrylated HA hydrogel

Hyaluronic acid methacrylate, HA-ma, is hyaluronic acid with a methacrylate group coupled to the backbone, see Figure 1 a). PhotoHA® from Advanced BioMatrix is a HA-ma that can be used for hydrogels with integrated cell culturing, in a system called 3D cell culture. When mixed with a photoinitiator and exposed to UV light, the photoinitiator emits free radicals that crosslink the vinyl bond on the edge of the methacrylate functionalisation of the hydrogel, forming a hydrogel (Choi *et al.* 2019). The origin of hyaluronic acid in PhotoHA® is from fermentation of *Streptococcus equi* susp. *zooepidemicus* (Bagley D 2017). Mesenchymal stem cells (MSCs) have previously been cultivated in this hydrogel (Lin S *et.al.* 2017) and thus a different category of cells (mouse brain microvascular endothelial cells bEnd.3 and human umbilical vein endothelial cells, HUVECs), which have not yet been studied, were chosen for investigation in this project.

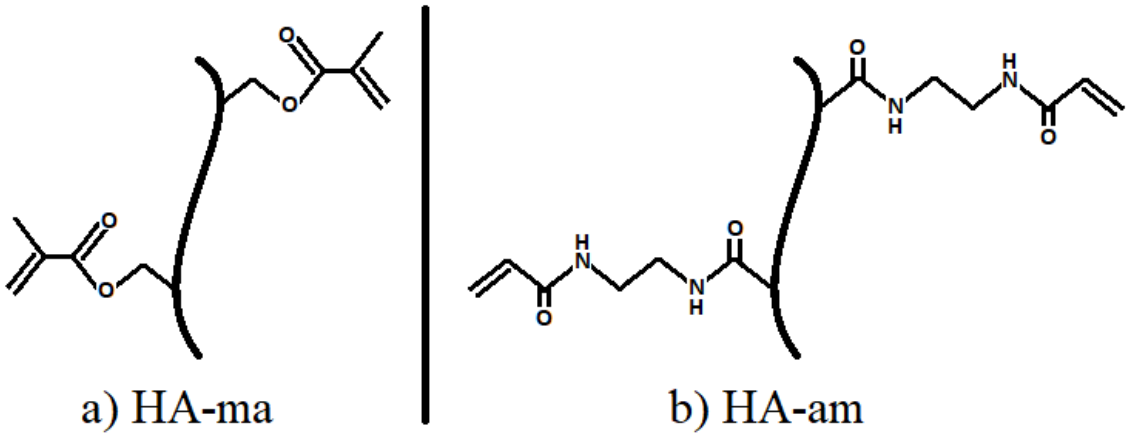

Figure 1. Representation of two different functionalisation of the hyaluronic acid backbone. a) Hyaluronic acid methacrylate (HA-ma) and b) hyaluronic acid acrylamide (HA-am).

1.2.3 Acrylamide HA hydrogel

Hyaluronic acid acrylamide (HA-am) is hyaluronic acid with an acrylamide group coupled to the backbone, see Figure 1 b). The HA-am used in this project was prepared according to previous protocol (Shi *et al.* 2017) and was a kind gift from Ana María in the lab group. HA-am is hyaluronic acid with acrylamide chemically linked to the backbone via carbodiimide coupling and thiol-disulfide exchange (Shi *et al.* 2017). In similarity to HA-ma, HA-am can also form a hydrogel by crosslinking of the acrylamide's vinyl groups (Choi *et al.* 2019, Porras Hernández 2022). This hydrogel has previously been used to show that bEnd.3 cells can successfully sprout on 2D when the surface is functionalised with RGD. During this project, we have investigated whether these cells are able to sprout under the same conditions, but in 3D instead of 2D.

1.2.4 Other types of hydrogels

As previously mentioned, HA does not promote cell adhesion on its own, instead it requires the addition of cell adhesion proteins. Kessler *et al.* (2017) mixed gelatine methacryloyl (GelMA), HA-ma, fibrinogen and thrombin in a hydrogel called QuattroGel, and encapsulated human adipose-derived stem cells, hASCs. The hydrogel mixture would then have sufficient binding sites for the cells to form tissue, and the QuattroGel proved to be a good candidate for accomplishing fatty tissue *in vitro* (Kessler *et al.* 2017). An additional experiment when cultivating human umbilical vein endothelial cells, HUVECs, on the QuattroGel was performed, where it was found that the QuattroGel enhanced proliferation of HUVECs compared to GelMA alone (Kessler *et al.* 2017). The origin of the idea to mix several different kinds of gels came from Aberle *et al.* (2014), who cultivated HUVECs in a QuattroGel containing of gelatin, transglutaminase, fibrinogen, and thrombin. HUVECs failed to form tubes on that QuattroGel but was still viable (Aberle *et al.* 2014). In this project, an idea of co-culturing two different cell type in a QuattroGel composed of GelMA, HA-ma,

fibrinogen and thrombin rose, which would improve the proliferation of HUVECs even more. The effects of co-culturing and its benefits will be discussed further on.

1.2.5 Crosslinking of hydrogel

As already mentioned, the hydrogel itself is formed after crosslinking of the polymers. The crosslinking process must be performed in a way that is non-toxic for the cells in order for them to survive and for the hydrogel to be stable (Tenje *et al* 2020). There are several different ways to crosslink a hydrogel, for example via heat, chemistry or via photopolymerization, the latter of which was chosen for this project. In photopolymerization a photoinitiator is irradiated by UV light, the photoinitiator absorbs the photons which promote the cleavage and creates free radicals. These free radicals react with the double bonds of the vinyl groups of the functionalization, leading to chemical crosslinks and hydrogel polymerisation (Choi *et al.* 2019). When cultivating cells in 3D using photopolymerization, the cells need to be able to handle the photoinitiator itself, the free radicals, and the UV light exposure for them to be successfully incorporated in the hydrogel.

Irgacure 2959 and lithium phenyl-2,4,6-trimethylbenzoylphosphinate, LAP, are two photoinitiators that are commonly used in 3D hydrogels due to their cytocompability. Irgacure 2959 absorbs UV light at wavelengths up to 365 nm, while LAP absorbs UV light at wavelengths up to 405 nm (Fairbanks *et al.* 2009). At shorter exposure times, wavelengths between these ranges do not harm cellular DNA and the cells can therefore be viable after exposure. According to previous studies published by Xu *et al.* (2020), cell viability is generally high up to 0.5 % Irgacure 2959 and up to 0.7 % LAP, but above these concentrations the photoinitiator becomes toxic for the cells. This also correlates to the length of UV exposure. With this background, concentrations up to 0.4 % Irgacure 2959 and 0.5 µg/µL LAP have been used in this study.

1.3 Primary cells and cell lines

In this project, four different types of cells have been encapsulated in different types of hydrogels, namely mouse brain microvascular endothelial cell line, bEnd.3; Mesenchymal stem cells, MSCs; Human Umbilical Vein Endothelial Cells, HUVECs; and Adult Human Lung Fibroblasts, hLFs. bEnd.3 is a cell line derived from mice, while MSCs, HUVECs and hLFs are primary cells from human. In Figure 2, the morphology of bEnd.3, HUVECs and hLFs when grown in 2D in flask is shown. What can be seen for all is the sprouting morphology that is expected in the hydrogel.

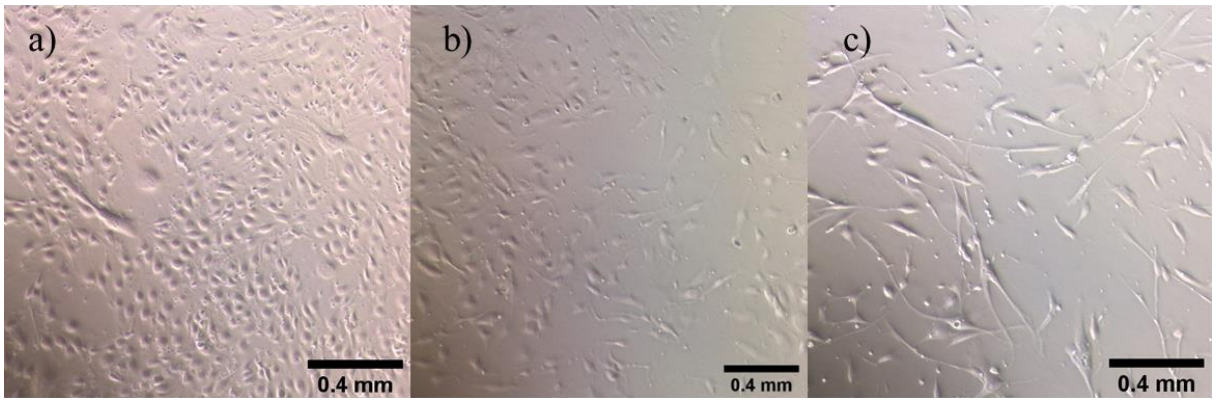

Figure 2. When grown in flasks sprouting cells can be seen. Brightfield pictures. a) bEnd.3 b) HUVECs c) hLFs.

1.3.1 Mouse brain microvascular endothelial cell line bEnd.3

The cell line mouse brain microvascular endothelial cells bEnd.3 was first established in 1990 by Montesano *et al.* and is now commercially available from ATCC®. The cell line is adherent, meaning that it sprouts, which can be seen in Figure 2 a). The cell line was obtained from mice with endothelioma by infecting a primary culture of brain endothelial cells from the cerebral cortices with a polyoma virus middle T-expressing N-TKmT retrovirus (Montesano *et al.* 1990). This inhibits the cell-cycle progression within the cells, making the cells immortalized (Rahman *et al.* 2016). Despite the immortalization, the company does not recommend using passages over 30 since some genetic differences appear at that stage (ATCC 2021).

In the brain, the cells make up the microvessels that controls what reaches the brain, meaning that one of the most important functions of the cells is the ability to form tight junctions. The tight junctions of bEnd.3 are one of the most characterised among endothelial cell lines, which makes it suitable for a model of the blood-brain barrier, BBB (Rahman *et al.* 2016). This is one of the reasons this cell line is interesting for cultivating in hydrogels and in 3D, because it makes it possible to study how for example pharmaceuticals are transported over the BBB (Reddy *et al.* 2021). Sprouting cells are the first step towards this vascularisation, and thus is this the ideal conformation of the cells, which is seen in 2D, see Figure 2 a), and this is desired in 3D as well.

1.3.2 Mesenchymal stem cells

Mesenchymal stem cells, or mesenchymal stromal cells, MSCs, can be derived from many different sources. The ones used in this project are primary cells derived from adult bone marrow, but MSCs can also be derived from umbilical cord, cord blood, placenta and different fetal tissues (Chou *et al.* 2013). MSCs has been successfully cultivated in HA-ma several times before (Poldervaart *et al.* 2017, Khetan & Corey 2019). One advantage for cultivating stem cells in 3D instead of 2D is the preservation of the stemness of the cells. Stemness is the capacity a stem cell has for self-renewal and differentiation. Previous studies have shown that MSCs maintained in HA-ma preserved the stemness of the cells, and they

could thereafter differentiate with differentiation media (Khetan & Corey 2019). Compared to maintenance in 2D, a cultivation in 3D can enhance MSCs differentiation potential (Cesarz & Tamama 2016). In addition, in 2D can the cells start to sprout even without the addition of differentiation media, meaning that an undirected differentiation has taken place. By keeping MSCs in hydrogels instead of regular 2D would then give a better cell maintenance, and more cells would stay in the wanted formation (Cesarz & Tamama 2016). For MSCs, the addition of functional peptides in the hydrogel, such as RGD, is not necessary and can even lead to a loss in viability (Khetan & Corey 2019). Previous studies have shown that in higher HA-ma concentrations MSCs shows a rounder morphology while in lower percentage more elongated cells are seen (Poldervaart *et al.* 2017).

1.3.3 Human Umbilical Vein Endothelial Cells

Human Umbilical Vein Endothelial Cells, HUVECs, are primary cells from healthy umbilical cord. Common applications of HUVECs are for 3D cell culture and for cardiovascular disease research. When grown in 2D, they have similar morphology as bEnd.3, seen in Figure 2 b), since they both are endothelial cells. HUVECs have been cultivated in different kinds of hydrogels before, like gelatin methacryloyl, GelMA, (Khayat *et al.* 2017) as well as a mixture of methacrylate gelatin, methacrylate hyaluronic acid, fibrinogen, and thrombin (Kessler *et al.* 2017). A wanted formation of HUVECs in hydrogels are vascularisation, which is the formation of tubes, which in turn can be used for tissue engineering (Gaihre *et al.* 2021). In similarity with bEnd.3, sprouting cells are a step towards this conformation, and are therefore the measure stock to a successful cultivation. Sprouting HUVECs inside hydrogel would shows both the scaffold optimum for the cells, as well as the potential to use hydrogels as a scaffold for tissues.

1.3.4 Adult Human Lung Fibroblasts

The chosen fibroblasts for this project were Adult Human Lung Fibroblasts (hLFs), which are primary cells from healthy normal human lung tissue. Fibroblasts are connective cells, which can be seen in their sprouting morphology in Figure 2 c), that secrete many of the molecules that make up the ECM of several different tissues, including that of the lung, heart, skin, and muscle (Plikus *et al.* 2021). Due to this nature of the cells, they are good candidates for co-culture with other cells, as they exclude the need of adding necessary growth factors. In previous study, co-culturing fibroblast and HUVECs were compared to addition of either Vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF), where it was found that co-culturing with fibroblast promoted microvessel formation of HUVECs (Sukmana & Vermette 2010). One explanation for this is since fibroblast release many growth factors, that stimulate the sprouting of HUVECs. By co-culturing cells in a hydrogel, the system takes one step closer to the natural environment and therefore also provide more reliable data for *in vitro* studies.

1.4 Aim

The aim of this project is to establish a protocol for culturing cells in hydrogel for further analysis of metabolome with mass spectrometry. To accomplish this, four types of cells were evaluated for growth in hyaluronic acid methacrylate (HA-ma), hyaluronic acid acrylamide (HA-am) as well as a QuattroGel composed by gelatin methacryloyl (GelMA), HA-ma, fibrinogen, and thrombin. Questions to be answered are:

- Can mouse brain microvascular endothelial cells bEnd.3 sprout in hyaluronic acid methacrylate hydrogel?
- Can mouse brain microvascular endothelial cells bEnd.3 sprout in hyaluronic acid acrylamide hydrogel?
- Can a replicate of mesenchymal stem cells in hyaluronic acid methacrylate hydrogel be done for future metabolome profiling?
- Can a replicate of human umbilical vein endothelial cells in a QuattroGel be done for future metabolome profiling?

2 Materials and methods

In Table 1 the used materials in this study are listed.

Table 1. Materials used in the experiments.

Category	Product	Company
Cells	Brain endothelial cells bEnd.3	ATCC
	Human umbilical vein endothelial cells	
	Mesenchymal stem cells	Uppsala University Hospital
	Adult human lung fibroblasts	PELOBiotech GmbH
Hydrogels	PhotoHA®	Advanced BioMatrix
	Gelatin methacryloyl	Sigma-Aldrich
	Fibrinogen	
	Thrombin	
Photoinitiator	Irgacure 2959	
	LAP	
Peptides	5FAM-GCG with sequence 5FAM-GCGYRGDSPG-CONH2 (RGD-f)	Innovagen
	GCG10 with sequence NH2-GCGYRGDSPG-CONH2 (RGD-nf)	
Cell media	Gibco™ Dulbecco's Modified Eagle Medium (DMEM), High glucose, GlutaMAX™, with phenol red	Thermo Fisher Scientific
	Gibco Dulbecco's Minimum essential media (MEM) without phenol red	
	Gibco™ Sodium pyruvate	
	Fetal bovine serum (FBS)	
	D-glucose	Sigma-Aldrich
	Penicillin-Streptomycin Mixtures	Lonza
	Cellovations® Fibroblast Basal Medium w/o Glutamine	PELOBiotech
	Cellovations® Endothelial Cells Growth Medium kit classic (GFP/RFP)	
	Endothelial Cell Growth Supplement Kit classic	
Coating	Speed Coating Solution	PELOBiotech
Enzymes	TryplE	Thermo Fisher Scientific
Other chemicals	Dulbecco's Phosphate buffered saline	Fisher Scientific
	Triethylamine anhydrous	Sigma-Aldrich
Stains	Calcein AM	Thermo Fisher Scientific
	Propodium Iodide	
Equipment	Glass cover slips 18 mm * 18 mm	VWR
	UV LED 365 nm Curing lamp, 100 W	Tao Yuan Electron

A summary of the workflow is shown in Figure 3. The steps will be described in more details further down, but in short, the cells are collected from the flask, all components of the hydrogel are prepared and mixed, and at last the hydrogel precursor is added to a cover slip with a PDMS frame and crosslinked with UV light until gelation. Cell media is added, and the hydrogel is incubated at 37 °C with 5 % CO_2. All experiments and maintenance of cells were conducted in NinoSAFE Biological Safety Cabinet CLASS II Pro to ensure sterile conditions.

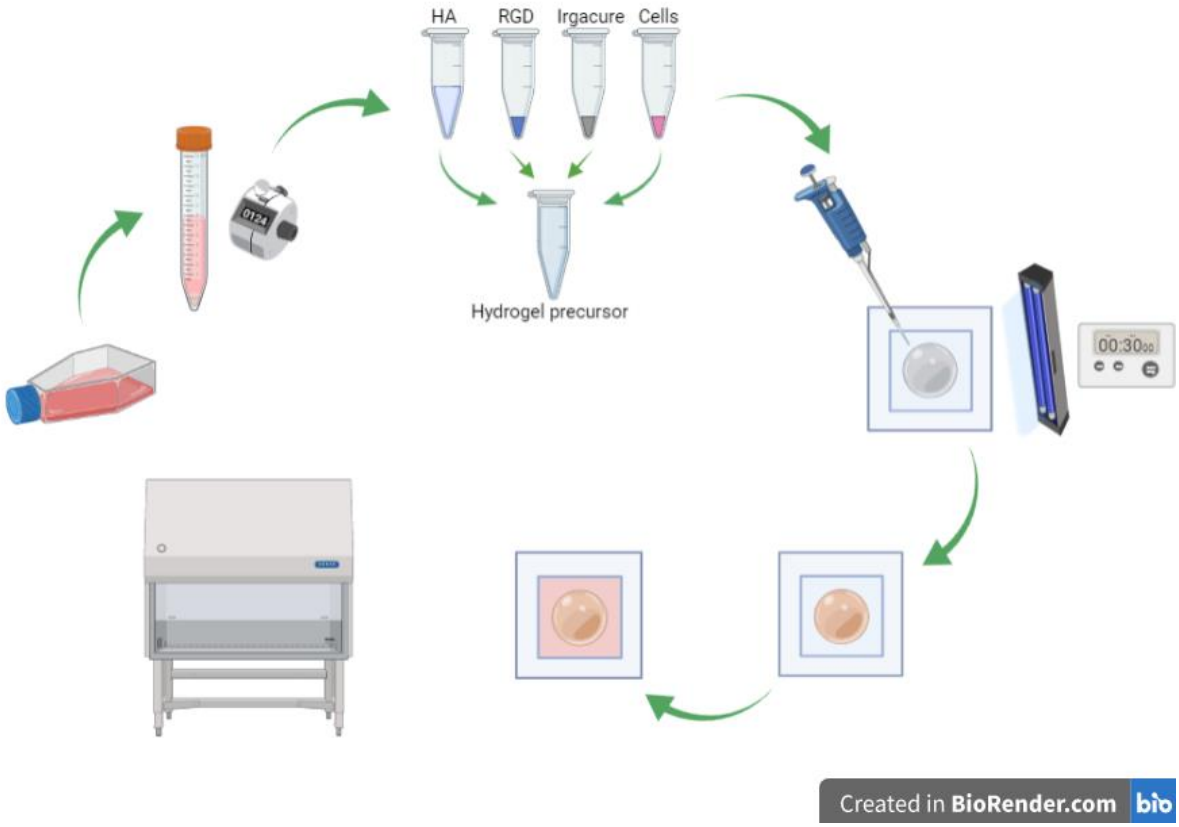

Figure 3. Overview of the hydrogel fabrication. First the cells are detached from the flask, counted, and resuspended in an appropriate volume. The components for the hydrogel precursor are prepared. The hydrogel precursor is then added to a coverslip with a PDMS frame and exposed to UV light until gel-like. Appropriate media was added and the hydrogel was incubated at 37 °C with 5 % CO_2. Created with BioRender.com.

2.1 Cell maintenance

Mouse brain microvascular endothelial cell line bEnd.3 was cultured in Gibco Dulbecco's Modified Eagle Medium (DMEM) high glucose supplemented with pyruvate, glutamine, phenol red, supplemented with 10 % fetal bovine serum (FBS) and 1 % Penicillin-Streptomycin. The cells were maintained at 37 °C with 5 % CO_2. The cells were either passaged or used in experiments at 80-100 % confluency. Passages between 23 and 30 were used in all experiments. Media was changed every second to third day to ensure good maintenance.

Human Umbilical Vein Endothelial Cells were cultured in Cellovations® Endothelial Cells Growth Medium kit classic (GFP/RFP) supplemented with Endothelial Cell Growth Supplement Kit classic. The cells were maintained at 37 °C with 5 % CO_2 in flasks coated with Speed Coating Solution to enhance adherence. The cells were either passaged or used in experiments at 70-90 % confluency. Passages between 5 and 13 were used in all experiments. Media was changed every second to third day.

hLFs were cultured in Cellovations® Fibroblast Basal Medium w/o Glutamine. The cells were maintained at 37 °C with 5 % CO_2 in flasks coated with Speed Coating Solution to enhance adherence. The cells were either passaged or used in experiments at 70-90% confluency. Passages between 5 and 12 were used in all experiments. Media was changed every second to third day.

Mesenchymal stem cells were cultured in Gibco Dulbecco's Modified Eagle Medium (DMEM) high glucose with pyruvate, glutamine, phenol red, supplemented with 10 % fetal bovine serum (FBS) and 1 % Penicillin-Streptomycin. The cells were maintained in 37 °C with 5 % CO_2. The cells were either passaged or used in experiments at 70-90 % confluency. Passages between 5 and 6 was used in all experiments. Media was changed every second to third day.

2.2 Hydrogel fabrication

For the preparation of the hydrogel, several components were prepared across several steps.

2.2.1 Preparation of glass cover slip with PDMS frame

Glass cover slips and ready cut out frames were plasma treated for 30 s and then mounted together. Before use, the cover slips were wiped with ethanol and UV irradiation for 10 min using NinoSAFE Biological Safety Cabinet CLASS II Pro.

2.2.2 Cell preparation

The cells were first rinsed with phosphate buffered saline (PBS) and then disassociated by TrypLE with 3 min (HUVECs and hLFs) or 5 min (bEnd.3 and MSCs) incubation. Appropriate media was added to inhibit TrypLE, and the cells were centrifuged for 5 min at 1000 x g. The supernatant was removed, and the cell pellet was dissolved in appropriate media, without phenol red. The number of cells was counted using EVE™ Automated Cell Counter, NanoEnTek.

2.2.3 HA-ma hydrogel precursor

PhotoHA® from Advanced BioMatrix were prepared as recommended from the provider. In short, the lyophilized methacrylated hyaluronic acid (HA-ma) powder was dissolved in appropriate media in an Eppendorf tube, to concentration of 1 %, 2 %, 2.5 % or 3 %. The solution was vortex until dissolved, around one hour. The precursor was either used immediately or incubated overnight with 1 mM RGD at 37°C in a heating block. To form the ready hydrogel precursor, Irgacure 2959, cell suspension and RGD was added and mixed thoroughly before the hydrogel was transferred to a glass cover slip with a PDMS frame in appropriate volume.

2.2.4 Crosslinking of the hydrogel

Crosslinking of the hydrogel was done via a photoinitiator and a UV light source. Irgacure 2959 was dissolved in methanol to a 10 % stock solution. The UV source used was mounted

on a 10 cm high holder, which gives the UV dose 0.2 J/cm^2 per second in a linear regime, meaning that extended time resulted in higher energy. Evaluation method to see if the hydrogel was crosslinked was to carefully touch the hydrogel with a pipette tip as well as attempts to pipette the hydrogel. The hydrogel was exposed to UV light until it was no longer able to pipette.

2.2.5 HA-am hydrogel preparation

A hydrogel protocol that had already been established in the lab used for 2D was tried for encapsulating cells. 10^6 cells/mL was encapsulated in 2 % acrylamide modified HA hydrogel, HA-am, with 0.4 % Irgacure 2959 and 1 mM RGD-nf. The hydrogel size was either 80 µL, 50 µL, 30 µL or 20 µL and the UV exposure was either 27 s or 30 s. The cells were either HUVECs prepared in 96-well plate, or bEnd.3, on cover slip. One hydrogel was prepared without RGD-nf and HUVECs were added.

2.2.6 Preparation of QuattroGel

If nothing else specified, all components were dissolved in PBS. Stock solutions of 10 % w/v GelMA and 2.5% w/v HA-ma was prepared and stored in 4 °C no longer than overnight. Before use, the hydrogel precursor was warmed up in 37 °C. Fibrinogen stock solution of 32 mg/mL and thrombin stock solution of 0.4 U/mL was prepared. A stock solution of 10 µg/µL LAP was prepared. Final concentration in the QuattroGel was 4 % GelMA, 1% HA-ma, 0.16 mg/mL fibrinogen, 0.02 U/mL thrombin, 6 % v/v triethylamine (TEA) and 0.5 µg/µL LAP. The cell concentration used was either $1*10^6$ cells/mL or $2*10^6$ cells/mL. QuattroGel volume was between 20 µL and 60 µL. The QuattroGel was crosslinked with UV LED 365 nm Curing lamp, Tao Yuan Electron, mounted on a 10 cm holder for 10 to 30 s. The QuattroGel was washed 3 times with media before fresh media was added. Two different experiments with the QuattroGel were done, one with only HUVECs and one with co-culturing of HUVECs and hLFs. For the co-culturing, a ratio of 2:3 (HUVECs:hLFs) was used, with a maintained cell concentration of $1*10^6$ cells/mL.

2.2.7 Evaluation of cell viability/ Calcein-am propodium iodide staining

During the experiments, the evaluation procedure used to determine whether the cells were sprouting or not was performed by inverted microscopy (Nikon Eclipse TE2000-U). A Live/Dead staining with Calcein AM and Propodium Iodide was carried out in order to see if the encapsulated cells were dead or alive. The hydrogel was washed one time with DMEM transparent media. Calcein AM and Propodium Iodide was added to DMEM transparent in final concentrations of 4 µM and 100 µg/mL respectively. The hydrogels were covered in the solution and incubated in 37 °C, 5 % CO_2 for 30 min. The hydrogels were then washed three times with DMEM transparent. Pictures were taken with a laser confocal microscope (Leica SP8).

2.2.8 hLFs in fibrinogen and thrombin

hLFs cultured in fibrinogen and thrombin was performed according to a protocol already established in the lab. All components were dissolved in PBS in order not to disturb the

crosslinking. 5 mg/mL fibrinogen was mixed with 0.5 U/mL thrombin together with 700 000 hLFs/mL. The solution was mixed thoroughly and 100 µL hydrogel precursor were added on a glass coverslip with a PDMS frame, and media was added on top of the hydrogel and around the hydrogel. The hydrogel was kept in 37 °C, 5 % CO_2.

3 Results

3.1 MSCs can stay alive in HA-ma hydrogel

Mesenchymal stem cells can stay alive 8 days in 1 % HA-ma hydrogel without RGD, with 0.05 % Irgacure 2959 crosslinked with 10 min UV light. In Figure 4 a), sprouting cells can be seen, and in Figure 4 b) a confocal picture of HA-ma without RGD is shown, where the green spots represents live cells. The dark areas in the middle of the hydrogel are a crack in the hydrogel, which were where the sprouting cells was found. After Calcein AM and Propodium Iodide staining, sprouting cells were no longer visible, suggesting that they had adhered to the glass cover slip and not in the hydrogel. This shows that the cells were alive in the hydrogel and kept the round formation, while when not in the hydrogel, the cells start to sprout.

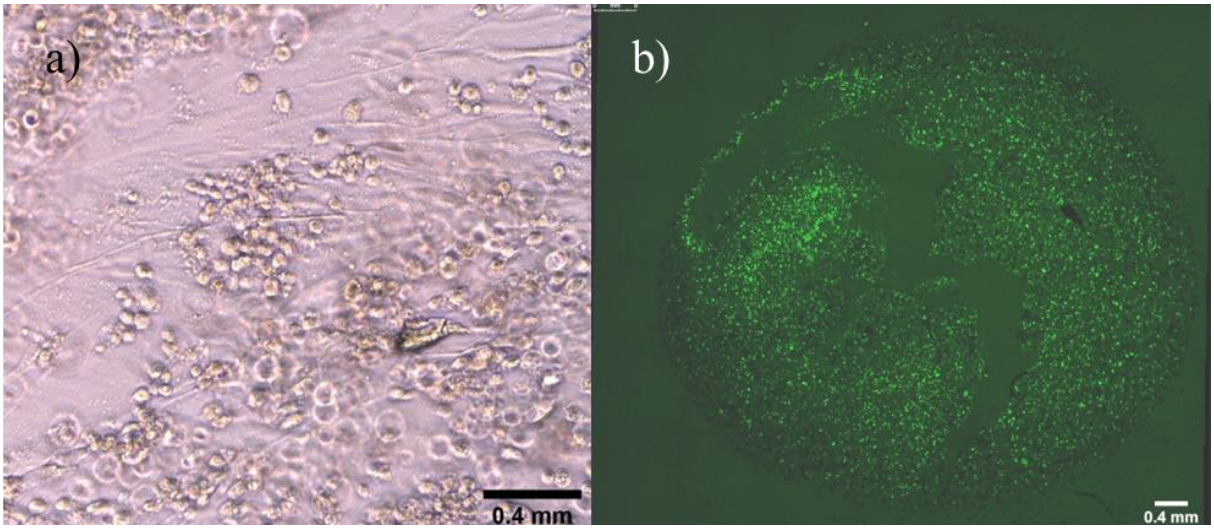

Figure 4. MSCs are maintained in a round conformation in 1 % HA-ma hydrogel with 0.05 % Irgacure 2959 crosslinked with 10 min UV light. Cell concentration $5*10^5$ MSCs/mL. a) Sprouting cells can be seen in areas outside the hydrogel. Brightfield picture. b) Round cells can be seen, while the sprouting cells have detached. Confocal picture.

3.2 bEnd.3 cannot sprout in 3D methacrylate modified HA hydrogel

Several attempts with growing bEnd.3 in 3D in HA-ma hydrogel was done. It was possible to confirm that the cells had been encapsulated in the hydrogel through microscopy, by seeing

cells in stacks. However, the cells stayed round in the hydrogel, even after several days, see Figure 5 a). Fresh media was added every second to third day. Since bEnd.3 cells sprout in the native environment, round cells are not considered to be a desired morphology. In a Table 2 a summary of the tested variables and what did not generate elongating cells are listed.

Table 2. Summary of tested variables of HA-ma hydrogel. DMEM = Dulbecco's Modified Eagle Media supplemented, HG = high glucose, LG = low glucose, PSP = phenol red, PBS = phosphate buffered saline, RGD-f = fluorescent RGD-peptide, RGD-nf = non-fluorescent RGD peptide. UV exposure was given in intervals of 8 s, 10 s or 19 s with 5 s rest between.

Hydrogel volume, µL	**HA concentration % w/v and solvent**	**Irgacure 2959 concentration**	**Cell concentration, cells/mL**	**RGD type and concentration**	**UV exposure time**
300	1 %, DMEM HG PSP	0.1 %	120 000	-	76 s
320	1 %, PBS	0.4 %	120 000	-	19 s and 24 s
320	3 %, PBS	0.1 %	120 000	-	305 s
50	1 %, PBS	0.4 %	120 000	-	16 s
50	1 %, DMEM LG	0.2 %	120 000	-	139 s
50	1 %, DMEM LG	0.3 %	120 000	-	64 s
300	1 %, DMEM LG	0.4 %	1 000 000	-	90 s, 20 s
50	1 %, DMEM LG	0.4 %	1 000 000	0.5 mM RGD-f	28 s
50	1 %, DMEM LG	0.4 %	1 000 000	1 mM RGD-f	18 s
50	1 %, DMEM LG	0.4 %	1 000 000	1.5 mM RGD-f	20 s
50	1 %, DMEM LG	0.4 %	1 000 000	2 mM RGD-f	20 s
150	1 %, DMEM LG	0.4 %	1 000 000	0.5 mM RGD-f	28 s
150	1 %, DMEM LG	0.4 %	1 000 000	1 mM RGD-f	20 s
150	1 %, DMEM LG	0.4 %	1 000 000	1.5 mM RGD-f	28 s
150	1 %, DMEM LG	0.4 %	700 000	0.5 mM RGD-f	28 s
150	2.5 %, DMEM HG, incubated ON with RDG	0.05 %	1 000 000	1 mM RGD-f	50 s
150	2.5 %, DMEM HG, incubated ON with RDG	0.05 %	1 000 000	1 mM RGD-nf	180 s
20, 30, 50	2 %, DMEM HG	0.4 %	1 000 000	1 mM RGD-nf	30 s

Two times during the project bEnd.3 have been sprouting in a hydrogel environment. First time was with a hydrogel precursor containing of 1 % HA-ma, 0.1 % Irgacure 2959, dissolved in DMEM PHS and 120 000 cells/mL, that had not been crosslinked. The second time was a hydrogel with 1 % HA-ma, 0.4 % Irgacure 2959, dissolved in DMEM LG and 120 000 cells/mL, with 16 s UV exposure. In the latter case, it was found that the cells had adhered to the cover slip and not encapsulated inside the hydrogel, by removal of the hydrogel. In Figure 5 b) it can be possible to see that the sprouting cells are below the round cells and attaching to the glass cover slip.

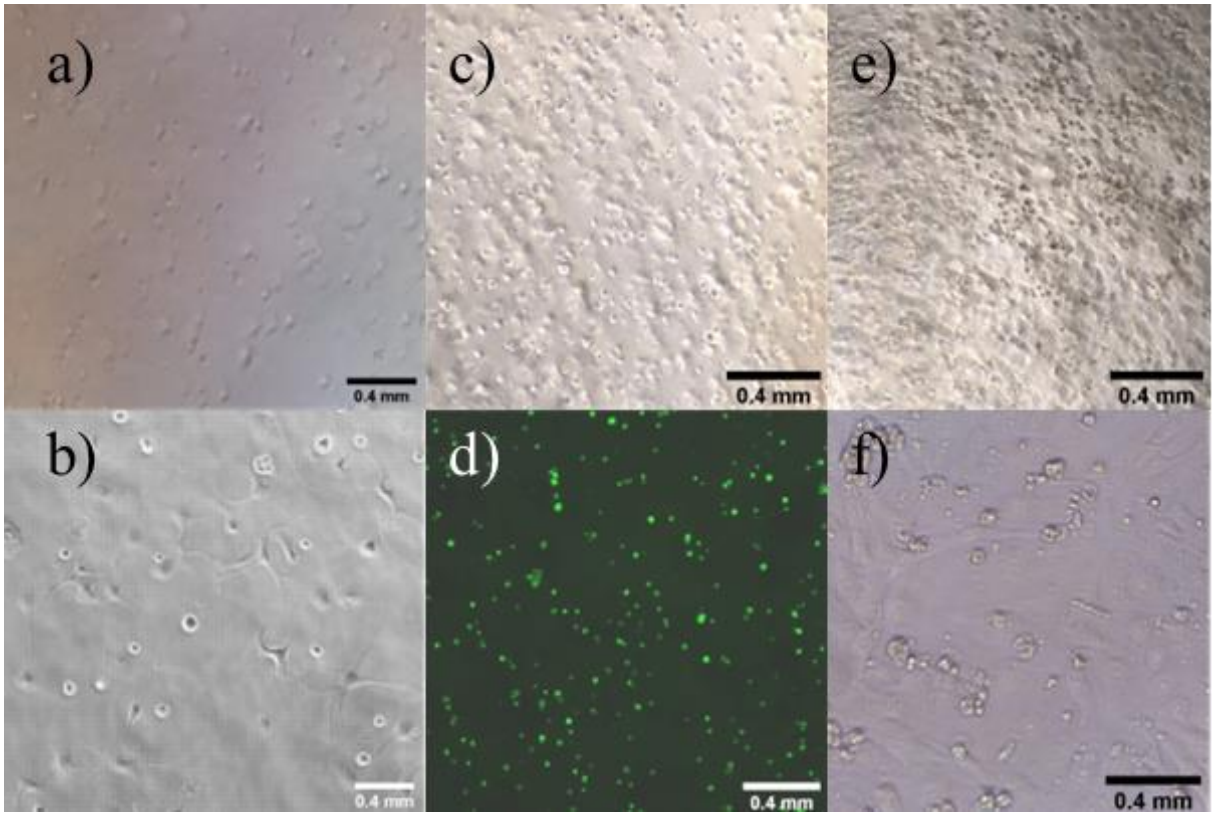

Figure 5. Cell growth in HA-ma and HA-am. a) Brightfield picture of 10^6 bEnd.3/mL, 2 % HA-ma, 0.4 % Irgacure 2959, 1 mM RGD-nf, 50 µL, 30 s UV exposure, 6 days in hydrogel. b) Sprouting bEnd.3 can be seen. Brightfield picture of 120 000 bEnd.3/mL 1 % HA-ma, 0.4 % Irgacure 2959, 16 s UV exposure, 3 days in hydrogel. c) Brightfield picture of 10^6 bEnd.3/mL, 2 % HA-am, 0.4 % Irgacure 2959, 1 mM RGD-nf, 20 µL, 3 days in hydrogel. d) Confocal picture of 10^6 bEnd.3/mL, 2 % HA-am, 0.4 % Irgacure 2959, 1 mM RGD-nf, 20 µL, 5 days in hydrogel. e) Brightfield picture of 10^6 HUVECs/mL, 2 % HA-am, 0.4 % Irgacure 2959, 1 mM RGD-nf, 80 µL, 5 days in hydrogel. f) Sprouting HUVECs can be seen. Brightfield picture of 10^6 HUVECs/mL, 2 % HA-am, 0.4 % Irgacure 2959, 50 µL, 5 days in hydrogel.

3.3 Endothelial cells cannot sprout in 3D acrylamide modified HA hydrogel

HUVECs and bEnd.3 was encapsulated in HA-am hydrogel according to a protocol already used for 2D structures. bEnd.3 were only round in HA-am, see Figure 5 a) and Figure 5 c). In Figure 5 d), a confocal picture from the Calcein AM and Propodium Iodide staining of bEnd.3 in HA-am is shown. There we can see live cells, suggesting that the cells can handle the treatment but are unable to sprout. After 5 days, the HUVECs were still round in the hydrogel, see Figure 5 e). In the hydrogel without RGD the cells had adhered to the bottom of the plate and started to sprout, suggesting that the environment is not toxic and that the cells are alive, see Figure 5 f). On all hydrogels, cells could be seen in the surrounding media, even though the hydrogels had been washed, suggesting that the crosslinking was not enough. On the contrary, the hydrogels exposed to 40 s of UV light broke at touch, suggesting a fragile

hydrogel. In all hydrogels, it was possible to see that the cells were encapsulated in the hydrogel.

3.4 QuattroGel

QuattroGel with a volume of 20 µL and cell density of $3*10^6$ HUVECs/mL were crosslinked with 20 s UV exposure, see Figure 6 a). Despite that, after staining with Calcein AM and Propodium Iodide few cells were visible, with live cells in green, see Figure 6 c). In Figure 6 b) the poking in the QuattroGel is shown. The QuattroGel has 0.5 µg/µL LAP and have been crosslinked with 30 s of UV light. As can be seen, there are some cell viability, but the cell concentration is very low. In similarity with other tested hydrogels, the cells are round. In Figure 6 d) a QuattroGel with $2*10^6$ cells/mL is shown.

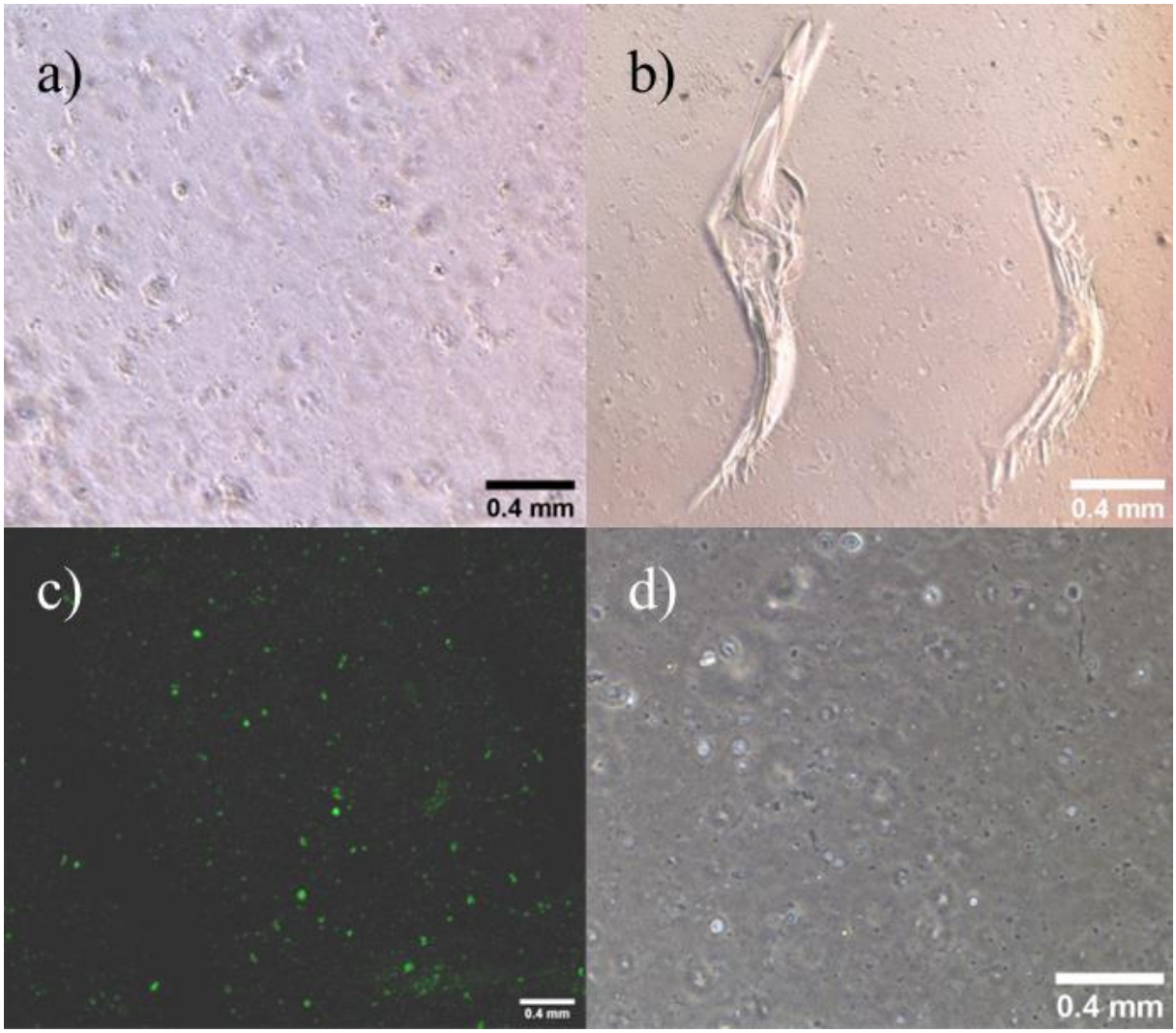

Figure 6. QuattroGel made of 4% GelMa, 1% HA-ma, 0.5 µg/µL LAP, 6 % v/v TEA, 0.16 mg/mL fibrinogen, 0.02 U/mL thrombin. a) Brightfield picture of $1*10^6$ HUVECs/mL + $4*10^4$ HUVECs, 20 µL, 3 days in QuattroGel. b) Marks after the pipette tip in the gel. Brightfield picture of $4*10^5$ HUVECs/mL, $6*10^5$ hLFs/mL, 20 µL, 1 day in QuattroGel. c) Confocal picture of $4*10^5$ HUVECs/mL, $6*10^5$ hLFs/mL, 20 µL, 11 days in QuattroGel. d) Brightfield picture of $2*10^6$ HUVECs, 20 µL, 2 days in QuattroGel.

3.5 hLFs in fibrinogen and thrombin

As expected, hLFs sprouted in 5 mg/mL fibrinogen and 0.5 U/mL thrombin, see Figure 7 a). Cell concentration encapsulated was the 700 000 hLFs/mL. Sprouting cells could clearly been seen throughout the hydrogel, as well as in stacks. Even one hour after gelation, it was possible to see a sprouting cell, picture not shown. After 7 days in hydrogel, more growth could be detected, see Figure 7 b).

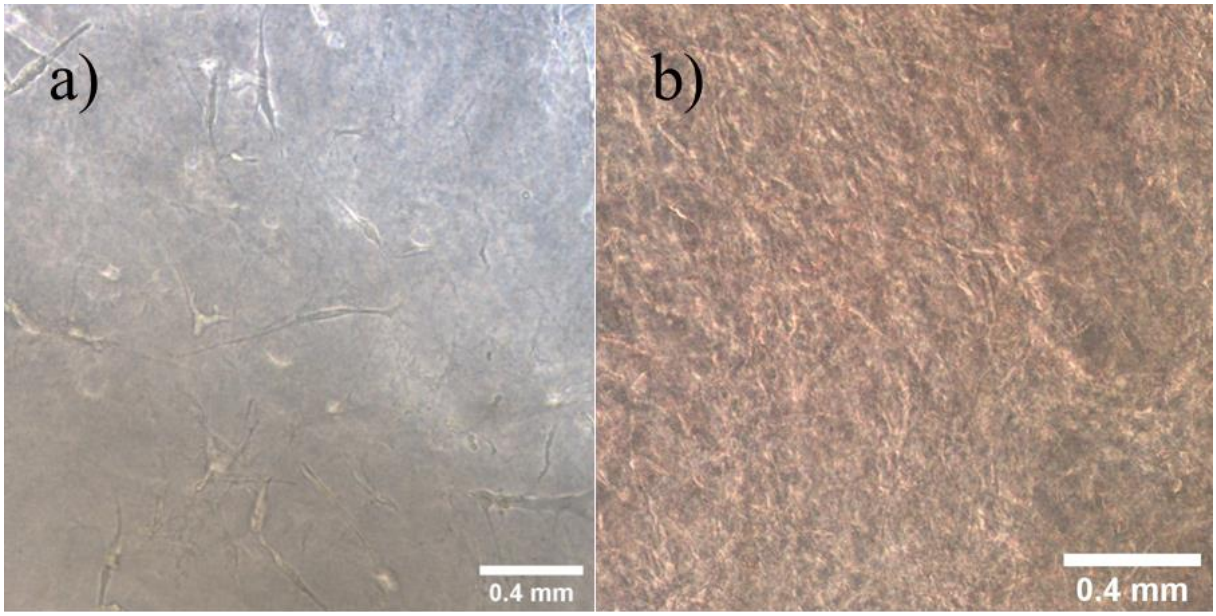

Figure 7. Brightfield picture of 700 000 hLFs/mL in 5 mg/mL fibrinogen and 0.5 U/mL thrombin. a) Picture after 2 days in hydrogel. Sprouts are clearly visible. b) Picture after 7 days in hydrogel.

4 Discussion

The original plan of the project was to characterize the hydrogel and to do a metabolome study of the cells encapsulated in the hydrogel. Due to difficulties in cultivating the cells in the hydrogel and to time constrains the metabolome study was excluded from the project. Instead, several different cell types were tested in several different types of hydrogels, to see if any supported sprouting cells. In this project no such combination was found, but it was found that cells were viable inside the hydrogel. This is therefore the first step towards cultivating cells in hydrogel and doing a metabolome study.

4.1 MSCs in HA-ma and HA-am

As previous experiments have shown, MSCs can survive in HA-ma and HA-am, both with and without the addition of RGD (Poldervaart *et al.* 2017, Khetan & Corey 2019). The present study shows that MSCs can stay in a round formation over a week in 1 % HA-ma with the hydrogel intact, while previous studies have reported 1 % HA-ma dissolving after 14 days in culture (Poldervaart *et al.* 2017). This study further suggests that HA hydrogel is a good scaffold for MSCs maintenance, and lower concentration of HA-ma is sufficient for maintenance for a shorter period. A lower HA concentration is advantageous for several reasons; use of less material provides a cheaper procedure, and a lower polymer concentration is easier to handle. In this study, HA-ma solutions up to 3 % were prepared, where the higher concentration of polymer significantly increased the time to solve the polymer, as well as the solution sticking to pipette tip, causing loss in material. Consequently, lower HA concentration is preferrable, and this study suggest that 1 % HA-ma is sufficient to maintain MSCs for a shorter period of time. The hyaluronic acid methacrylate used in this project, PhotoHA®, is commercially available, making it easy to start with. This have hence the potential for easy adaptation.

One problem that is present when maintaining MSCs in flasks is the loss of stemness, due to undirected differentiation (Cesarz & Tamama 2016). The advantage with stem cells is lost when undirected differentiation happens, since the whole idea of stem cells is to control the differentiation. To be able to use the full potential of stem cells, and MSCs in particular, the stemness needs to be maintained. This is a need that is not met when cultivating in 2D. Due to this point, it is crucial to develop a straightforward approach to maintain stemness within MSCs. In a further perspective, MSCs could be maintained in HA hydrogels instead of in regular flasks, and the ratio of cells with maintained stemness could possibly increase.

A sign of the advantage of cultivating the cells in hydrogel instead of in plate, is that MSCs started to sprout in areas outside the hydrogel. The unwanted differentiation was immediate outside the hydrogel, see Figure 4 a). In Figure 4 b), a confocal picture from Calcein AM and Propodium Iodide staining made of the same hydrogel is shown, which also proves that the cells are alive and can survive UV exposure for 10 min. Since the cells are alive both inside

the hydrogel and outside, but have the wanted conformation inside the hydrogel, this proves the advantage for hydrogels over flasks.

With hydrogels, instead of detaching the cells from the flask, the hydrogel needs to be degraded. In order to degrade a HA hydrogel, hyaluronidase is needed. It is an enzyme, endoglycosidase, that breaks down the glycosidic bonds in the hyaluronic acid to monosaccharides and should therefore not affect the cells (Jung 2020). For evaluation of the effect of hyaluronidase on MSCs, MSCs alone would be needed to be exposed for the hyaluronidase to confirm that the cells could handle it. The cells could then be passaged into new hydrogels instead of new flasks, with possibly more maintained stemness.

For further characterisation of how the cells grow in hydrogel versus in flask, a metabolome study can be done. The metabolome is all small molecules, metabolites, present in a cell or a tissue during a certain time point and under certain conditions (Nelson DL & Cox MM 2005). A metabolite can be everything from metabolic intermediate to signal peptides and typically have a mass below 1000 Da (Nelson DL & Cox MM 2005). By definition, if the environment changes in the cell or around the cell, it is a great chance that the metabolome change. Therefore, this have the possibility to give a good indication of how the cells are affected by the hydrogel environment. To compare the difference between culturing cells in flasks to hydrogels and to see the difference between hydrogel and the native environment would further prove the advantage of hydrogels compared to regular 2D culturing. As already mentioned, the stiffness of a scaffold is crucial for the cell growth, where the softer 3D environment can maintain stemness of MSCs better compared to the stiff 2D environment. In the same way, a too stiff hydrogel will then maintain the stemness less than a soft hydrogel (Poldervaart *et al.* 2017). In addition to further characterise MSCs growth in hydrogel, coupling cell growth and stemness to the metabolome would provide further insights in the effect of the maintenance of MSCs in hydrogels.

4.2 bEnd.3 in HA-ma and HA-am

Initially, it was expected that the cells would grow in the hydrogel, and little optimisation would be needed since bEnd.3 had previously been grown on the hydrogel in 2D. It was quickly realised that it was not true. In terms of outlook, there are a number of possible avenues to explore. Some of these aspects will be discussed in the further sections.

4.2.1 Possible problems with the crosslinking

When growing cells in 3D and crosslinking the hydrogel with the cells encapsulated, many things can go wrong. For example, at the crosslinking event, the free radicals produced by the photoinitiator can react with nucleic acids or proteins in the cell, leading to DNA damage or even cell death (Choi *et al.* 2019). One evaluation step toward optimising bEnd.3 in HA-ma could be to investigate how the cells react to the free radicals released by the photoinitiator.

During the course of this project, bEnd.3 started to sprout in a hydrogel environment once, where it was later found that the cells had adhered to the cover slip and not sprouting inside the hydrogel, see Figure 5 a). The hydrogel collapsed at touch, showing an insufficient crosslinking. This imply that the cells can handle up to 0.4 % Irgacure 2959 and 16 s of UV exposure, but the hydrogel had not crosslinked completely meaning that the UV exposure was too little. In all other cases the cells have stayed round, even when cells have leaked outside the hydrogel. In comparison, MSCs started to sprout outside the hydrogel, even after crosslinking. There are three possible things that could have happened. One is that the cells have died in the process, possibly by the release of free radicals. A Live/Dead staining with Calcein AM and Propodium Iodide was conducted, but the cell concentration was too low in HA-ma to be able to draw any conclusions whether the cells have died or not. In HA-am live cells could be found, but no sprouting cells was found. The other possible explanation is that the porosity of the hydrogel was too low, meaning that the hydrogel was too compact, so it physically did not promote sprouting. To evaluate the porosity further, the hydrogel could be characterised with Scanning Electron Microscope. The third is the length of the UV light exposure. According to a UV exposure test conducted, HUVECs and MSCs could stand several minutes of UV exposure, while bEnd.3 and hLFs were floating 1 day after and were considered dead. Previous data from the lab suggested that bEnd.3 could survive 30 s of UV exposure with the lamp used. The UV exposure listed in Table 2 is the UV exposure needed for the hydrogel to crosslink. Therefore, longer UV exposure time than what the cells can handle was used in some cases, in order to see when gelation had been successful. As expected, less time was needed to crosslink hydrogels with a higher photoinitiator concentration, since more free radicals will be formed that can crosslink the hydrogel. For these reasons, it is of suspicion that bEnd.3 can stand some release of free radicals, but not the amount needed to crosslink the hydrogel, but further evaluation is needed in order to know for sure.

It was found that the volume of the hydrogel was important for how much UV exposure was needed to form a hydrogel, where a smaller volume of hydrogel also required less time to crosslink. One possible explanation of this it that the hydrogel precursor itself affect how the UV light can penetrate in the hydrogel, and with more crosslinking the UV light is hindered to penetrate the hydrogel. The UV exposure time, volume of the hydrogel and the photoinitiator concentration is therefore crucial to form a hydrogel that are suitable for the cells.

One difficulty when working with hydrogels is to determine whether a hydrogel has been formed or not. Due to differences in UV sources, it was hard to compare the exposure time from the literature. The method used in this project was to carefully poke the hydrogel with a pipette tip. If the hydrogel was unable to be pipetted, the gelation was considered complete. In some hydrogels, for example hydrogels with 0.4 % Irgacure and crosslinked under 40 s UV exposure, it was possible to see marks made by the pipette tip under the microscope and hydrogels with 0.05 % Irgacure and 10 min UV exposure broke when touched. These findings suggest that the hydrogel had too many cross-linkages, which may have resulted in a too stiff

hydrogel. If the hydrogel is too stiff, the point with cultivating in a hydrogel disappears since one of the advantages of cultivating in hydrogel is that the environment has a Young's modulus closer to the native environment. For stiffness measurement, a rheometer can be used. As a continuation of this project, the hydrogel can be characterised with a rheometer and Young's modulus can be decided. This would give further insight in the characterisations of the hydrogel, as well as to how the stiffness compares to the native environment. It would also give insight in whether the porosity of the hydrogel is the reason that the cells did not sprout.

Another unexpected occurrence during the project was that the methacrylate in the hyaluronic acid was the cause of round cells. Therefore, some hydrogels with acrylamide hyaluronic acid were made as well to evaluate this. bEnd.3 cells did not sprout in either hydrogel, but confocal pictures showed more cells in HA-am hydrogel compared to HA-ma hydrogel. However, this do not necessarily need to be linked to the methacrylate on the hyaluronic acid but can also be due to difficulties when pipetting the cells. Further characterisation of the functionalisation of the hydrogel is needed towards knowing exactly what caused the problem.

4.2.2 Possible problems with RGD

As can be seen in Table 2, HA-ma hydrogels were formed both with and without adding RGD. According to the literature, HA hydrogels do not support cell adhesion by themselves, and therefore RGD was added in an attempt to promote cell adhesion and sprouting. One theory that bEnd.3 stays round in HA-ma in this project is that the RGD do not give sufficient number of binding sites. In previous work, RGD has been incorporated into the hydrogel using either UV light (Porras Hernández *et al.* 2020) or overnight incubation (Khetan & Corey 2019) and both approaches were tested but without success. After overnight incubation, the hydrogel precursor became cloudy, which made it hard to observe the encapsulated cells at a later stage. When the fluorescent peptide was added to the hydrogel, visible fluorescence was observed in the surrounding media of the hydrogel, suggesting that the peptide was not successfully incorporated in the hydrogel itself. This was seen for all concentrations tested, but less leakage was seen for the lower concentrations. One possible reason for this is that the UV dose is not enough for both crosslink the hydrogel and incorporation of the peptide. The cells can stand up to 30 s UV, so the concentration of RGD need to be in good ratio with what can be crosslinked with that UV dose. Another possible reason for round cells in the hydrogel is that RGD crosslinks with the HA-ma but is present in too low concentration. In this project, concentrations up to 2 mM was tested but without sprouting cells, and in previous studies where RGD have been used in hydrogel, 1 mM have been used (Khetan & Corey 2019). Therefore, the studies continued with 1 mM of RGD. Another theory is that the hydrogel provides too many binding sites for the cells to sprout, but with the background of hLFs in fibrinogen/thrombin, this is not a likely theory.

4.3 QuattroGel

As in the previous study, the cells were viable inside the QuattroGel. The shape of the cells was round, as can be seen in Figure 6 a) and Figure 6 b). This is a co-culture with HUVECs and hLFs, which shows that the cells stay alive. In the staining, however, no distinction between the two different cell types can be made. The only thing the staining is giving is thus that some cells in the QuattroGel are alive, but not whether both kinds of cells are alive or only one of them. In this QuattroGel the cell density is very low, as can be seen in Figure 6 c). This comes with a major problem, which is that a certain cell density is needed in order to get the important cell-cell interaction, which is the point with doing a co-culture. This was a problem that rose in several hydrogels, that despite the calculations and the careful and repeated workflow, the cell density seemed to be very low. As already discussed, the end volume of the hydrogel is very small for the crosslinking process to be as efficient as possible. One solution to this is to prepare bigger volume of hydrogel, to reduce the risk of pipetting error, which was a measure taken at later stages of the project.

$2*10^6$ HUVECs/mL was incorporated in a QuattroGel, with the same components and concentrations previously used, in order to ensure a sufficient number of cells. 20 s of UV exposure was used to polymerase the QuattroGel. After 2 days in the QuattroGel, cells had not started to proliferate. According to a UV exposure test conducted, HUVECs could survive several minutes of UV exposure. Consequently, it is likely that there is another explanation of why the cells do not sprout. In previous studies, HUVECs showed an elongated morphology but no sprouts, while on Matrigel, which was used as a positive control, HUVECs started to vascularise (Kessler *et al.* 2017). What have been done in this project is a replicate with the same result. However, this leads the way towards further optimisation of this. One advantage with continuing to optimise a QuattroGel instead of using Matrigel is the price. Matrigel is a pricy product, while the QuattroGel used in this project is cheaper. With the QuattroGel approach, it would also be possible to find the minimum number of components needed to achieve sprouting cells and vascularisation in an *in vitro* environment.

In the second trial, due to a calculation mistake, 5 µg/µL of LAP was added, instead of 0.5 µg/µL. At this high concentration, 10 s of UV irradiation was enough to form a very stiff hydrogel. According to previous studies (Xu *et al.* 2020), this is a way too high concentration, and as expected the cells did not grow.

4.4 hLFs in fibrinogen and thrombin

To see cells sprouting in a 3D environment, hLFs were cultivated in a hydrogel composed of 5 mg/mL fibrinogen and 0.5 U/mL thrombin. Sprouting cells with the same morphology as in 2D could be seen, compare Figure 2 c) and Figure 7 a). After 7 days in the hydrogel, more growth could be seen. One important aspect in the experiment is that the crosslinking is due to chemistry and not via photopolymerization. The experiment lacks UV crosslinking and

release of free radicals, as the other experiments conducted and therefore no more conclusions can be drawn from that than to see sprouting cells in a hydrogel.

4.5 Future applications

Hydrogels are the future in many ways, and for hydrogels to reach its full potential, more characterisation needs to be done. One way to further characterise the cell maintenance inside hydrogel is by doing a metabolome study. As already have been mentioned, hydrogels can be used both for regenerative medicine, where human tissue have been destroyed in an accident or similar, or in the pharmaceutical development to see the effect on human tissue without risking the harm of people. To use hydrogels in pharmaceutical development would also possibly reduce the need of animal models, which would make the process both easier and more ethic.

5 Conclusion

In this project, several different cell types and hydrogels were tested for growth similar to the native environment. Neither of the tested endothelial cells started to sprout in any hydrogel, namely bEnd.3 in hyaluronic acid methacrylate nor hyaluronic acid acrylamide, and HUVECs in neither hyaluronic acid methacrylate, hyaluronic acid acrylamide nor QuattroGel. All hydrogels, however, supported cell viability, so the possibility for sprouts is still there. There are several possible reasons for the lack of sprouts, and to be able to draw any conclusions more studies are needed. HA-ma supported MSCs viability and could be used for further characterisation of the hydrogel and of the metabolome of the cells.

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