Microstructuring of Hyaluronic Acid cell culture scaffolds

Frida Sjögren

Abstract

Animal models often fail to predict the efficacy and response to drugs in humans, and also raise ethical concerns as many experiments cause pain, suffering or distress in the animals used. Thus, there is a need to develop new and better *in vitro* models which can help reduce the need for animal experiments.

The goal of this work is to explore the possibilities of using micro-structured hyaluronic acid hydrogels as cell scaffolds for *in vitro* studies. Two different approaches have been investigated. The first approach is to pattern a photocrosslinkable hyaluronic acid and gelatin hydrogel using the well-known method of photolithography, providing cell culture scaffolds with tailor made topographies. The second approach is to chemically link RGD peptides, which provide cell adhesion sites, using a photoinduced reaction, thus allowing for photopatterned gels with RGD peptides present in specific patterns.

The two approaches are one step on the way towards providing cell culture scaffolds which show the same rich variability in physical, topological and biochemical properties as the natural extra cellular matrix does *in vivo*.

Till Jonas

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Sjögren, F. Porras Hernández, A.M. Shi, L. Ossipov, D. Tenje, M. (2017). Structuring Hyaluronic Acid Gelatin hydrogels with UV lithography for use as cell culture scaffold. *Manuscript.*

II Porras Hernández, A.M. Sjögren, F. Shi, L. Ossipov, D. Tenje, M. (2017). Photopatterning Hyaluronic Acid hydrogels with RGD-peptides for cell culture scaffolds. *Manuscript*.

Contents

Abbreviations

AFM	Atomic Force Microscopy
BBB	Blood Brain Barrier
BCA	Bicinchoninic Acid
ECM	Extracellular Matrix
ESEM	Environmental Scanning Electron Microscopy
HA	Hyaluronic Acid
IPN	Interpenetrating Polymer Network
PDMS	Polydimethylsiloxane
PEG	Poly(ethylene glycol)
pHEMA	Polyhydroxyethylmethacrylate
PVA	Poly(vinyl alcohol)
RGD	Arginine-glycine-aspartic acid
SEM	Scanning Electron Microscopy
TEER	Transendothelial Electrical Resistance
VSI	Vertical Scanning Interferometry

Introduction

Animals are used within science for research, development, and quality control within human medicine, veterinary medicine, dentistry and also for biological studies of fundamental nature. Within the European Union 11.5 million animals were used for experimental and other scientific purposes during 2011 [1]. 284 000 animals were used in Sweden for these purposes during 2014. Out of these almost 20 700 animals were subjected to procedures classified as "severe" [2]. These are "Procedures on animals as a result of which the animals are likely to experience severe pain, suffering or distress, or long-lasting moderate pain, suffering or distress. Procedures that are likely to cause severe impairment of the wellbeing or general condition of the animals " [3]. Comparisons between the effect of treatment on animals and humans in clinical trials can show poor agreement. For some human tissue types animal models may not correctly predict the clinical efficacy [4, 5]. There is thus a need to develop new experimental methods that do provide meaningful information for treatment of humans [5]. Also, results found in animal trials do often show poor reproducibility and factors such as differences in the living conditions of the animals have an impact on the result [6, 7].

Considering both the ethical concerns due to suffering of animals during experiments, and the fact that experiment on animals cannot always predict the effect in humans and often display poor reproducibility, there is a need to develop new, alternative experimental methods. In Sweden there is governmental support for research concerning alternatives to conventional animal testing based on the principles of the "three R's"; Reduction, Refinement and Replacement, following the regulation defined by the European Union more than 30 years ago (Directive 86/609/EEC)[8] and most recently updated in 2010 (Directive 2010/63/EU) [9]. The aim is to find methods to reduce the amount of animals used and the suffering they are subjected to and to develop alternative methods, such as *in vitro* models, where no animals are needed [10]. The three R's concept was first presented al-

ready in 1959 by W.M.S Russell and R.L. Burch in their text “The principles of humane experimental techniques” [11].

1. *In vitro* models

An *in vitro* study is a study of biological phenomena outside the organism in artificial environments [12, 13] and serve as a tool for studying the impact of biochemical compounds on cells or microorganisms.
For *in vitro* studies, cells are taken from the tissue of a donor and harvested under controlled conditions. In a primary cell culture cells are taken from the donor and used directly in an experiment. Primary cells can be used during a limited amount of cell divisions. It is also possible to establish continuous cell lines, normally from tumor tissue, which can be used during up to approximately 30 cell divisions. Cell lines are commercially available together with standardized culture protocols. Compared to primary cells, cell lines generally show a more rapid growth rate and do not require as careful handling, but can after many cell divisions fail to mimic the physiology of cells *in vivo* [14].

In vitro studies are an important step in drug development but also for understanding disease mechanisms. Compared to animal models the *in vitro* models can be more tightly controlled and are usually less expensive and less time consuming. The *in vitro* studies also allow for high-throughput studies of large numbers of combinations of experimental parameters [4].
Improved *in vitro* models could reduce the number of animals needed for animal testing. As human cells can be used it is possible to overcome the issue of species specific results and tests can also be more easily standardized. Improved *in vitro* models allow for more thorough pre-clinical tests of putative drugs which in turn can help reduce the duration, cost failure rate and risk of clinical trials [15].

1.1 Traditional *in vitro* models

The most commonly used *in vitro* model used for studies of mammalian cells is the 2D monolayer of cells [4]. Traditionally these studies have been performed in cell culture flasks and Petri dishes.

Although the traditional *in vitro* models have contributed immensely to biological research there are features of the biochemical and biophysical environment experienced by cells *in vivo* which cannot be recreated in the traditional cell culture flask. [4] These include shear stress due to fluid flow, mechanical strain [16], interactions between different cell types within a tissue, cell- extra cellular matrix (ECM) interactions, 3D topography [17] and varying chemical composition. Traditional *in vitro* substrates, such as the cell culture flasks and Petri dishes, thereby lack the features required to guide cells into the assembly of coherent tissue [15]. Neither do they support studies of interactions at a systemic level (between organs) [18].

1.2 Improved *in* vitro models, organ-on-chips

In order to overcome the inherent limitations of traditional *in vitro* models it is of importance to develop techniques that allow researchers to mimic the chemical and mechanical environment which cells experience *in vivo.*

In organ-on-chips the common idea is to mimic different features of human physiology in a more advanced way than what can be done in a conventional Petri dish or tissue culture flask. An organ-on-chip is a cell culture model with microfluidic channels in which the activities and physiological responses of an entire organ are simulated [19]. This is done by combining technology from the area of microfabrication with knowledge from traditional cell culture models. The organ-on-chips are fabricated from different materials, such as glass, silicon or PDMS (polydimethylsiloxane) which are materials traditionally used for microchip manufacturing. Traditional fabrication techniques such as UV-lithography, dry- and wet etching and casting are used. The ultimate aim is to build interconnected "organs" each represented by an organ-on-chip, to build a platform to study human biology in at a more systemic level in a "body-on-chip" or "human-on-chip" [18, 19].

One common approach is to fabricate microfluidic chips in which cells are grown and subjected to fluid flow, and thereby shear stress, at physiological levels [20, 21]. By fabricating microchips in soft materials such as PDMS it is possible to manufacture stretchable chips in which cells can be subjected to mechanical strain. In these microchips the cells are subjected to strain which e.g. mimics the strain experienced by cells in the lungs due to respiration (lung-on-a-chip) or in the

gut due to peristaltic movement (gut-on-a-chip) [16, 22]. It has been found that cyclic mechanical strain enhances the uptake, and accentuates toxic and inflammatory responses to silica nanoparticles in the lung [22]. In the gut, cyclic mechanical strain combined with shear stress induced by fluid flow, has been found to stimulate cells to spontaneously recapitulate the structure of the intestinal villi [16].
Improved *in vitro* models can also allow for studies of permeability. One approach is to fabricate a microfluidic chip with two microchannels separated with a permeable synthetic membrane on which cells are cultured. By introducing molecules, often dextran, into only one of the channels the exchange of molecules between the channels can be determined, by subsequently measuring the resulting concentration in the other channel. The resulting permeability value is used as a measurement of the barrier function of the cell monolayer. If electrodes are incorporated within the chip it is also possible to directly measure the Trans Endothelial Electrical Resistance (TEER) value which is also a measurement of the barrier function. The setup also allows for the cells being subjected to shear stress and contact co-cultures where the cells are kept separate in space but still in contact via chemical interactions. The barrier function previously has been shown to be influenced by both shear stresses and that co-culturing cell types (endothelial cells and astrocytes) allows for reestablishment of many Blood Brain Barrier (BBB) features [23]. As the understanding of the barrier function of the BBB is essential to develop new drugs to treat e.g. Alzheimer's disease this setup is a promising approach.
There are examples where several organ-on-chips have been connected in the step towards creating a "body-on-chip". These include e.g. multi-channel microfluidic chips where human liver, lung, kidney and adipose cells are cultured separately but connected to the same reservoir for cell culture media [24]. In other approaches cells representing human intestine, liver, skin and kidney [18], or skin and vasculature [25] have been combined. A "body-on-chip" provides a platform to study the absorption, distribution, metabolism and excretion of e.g. drugs, food substances and air pollutants on a systemic level.

1.3 Improved *in vitro* models, cell culture scaffolds

Apart from studying the impact of shear stress, mechanical strain and interactions between organs, microfabrication approaches also allow for tailor made cell culture scaffolds mimicking the ECM. The ECM

is a non-cellular component of all tissues and organs. It serves as an essential physical scaffold for cells and initiates biomechanical and biochemical cues required for tissue formation and preservation. The physical, topological and biochemical properties of the ECM vary not only between tissue types, but also shows heterogeneity within the tissues [26].

The materials chosen to create these cell culture scaffolds are generally either synthetic or naturally derived polymers. The 3D shape and the chemical composition of the scaffold can be defined on the microscale i.e. at the size scale of individual cells. Not only traditional microfabrication materials can be used, but also materials which mimic, or are natural constituents of the ECM.

One example of where the 3D shape of the cell culture scaffold plays an important role is when creating models of the small intestine. *In vivo* the absorptive cells (enterocytes) grow on finger like projections called villi, which increase the absorptive area of the small intestine [27, 28]. By using microfabrication techniques these structures can be re-created in cell culture scaffolds which allow the cells seeded on top of them to grow in a pre-defined shape.

These cell culture scaffolds, unlike flat surfaces without any topography resembling the villi, have been found to cause epithelial cell morphology and differentiation to be more resembling to that of native intestinal tissue [29]. Also, a more villi-like structure has been shown to stimulate expression of various mucin proteins which in turn has an effect on the ability to provide protection from bacterial infection [30].

The cell culture scaffolds have been fabricated in e.g. SU-8 by air drying thin membranes over silicon pillars, leaving a structure resembling that of the villi [31]. Also, materials present in the natural ECM can be used to form villi-like structures. Many groups have used collagen, which can be cast using a combination of PDMS soft lithography and a sacrificial calcium alginate mold [17, 30]. The process involves several steps, Figure 1. First, a plastic mold is created by e.g. laser ablation and is used to cast PDMS mold. The next step is to cast a sacrificial calcium alginate mold in the PDMS mold. Finally the collagen is cast on the calcium alginate mold. The calcium alginate is then dissolved leaving only the collagen structure.

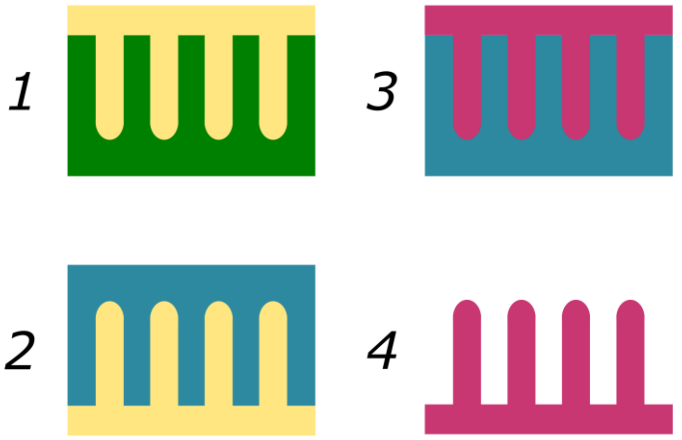

Figure 1 Casting of collagen using PDMS and an alginate sacrificial mold. The fabrication process involves (1) casting of PDMS (yellow) in a plastic mold (green), (2) casting of a sacrificial alginate mold (blue) using the PDMS mold. The collagen (red) is then cast in the alginate mold (3) and as the alginate mold is dissolved leaving only the collagen structure (4).

Cell culture scaffolds with defined micro-topographies can also be defined using computer-based techniques such as 3D printing or multiphoton lithography. These techniques are however often based on synthetic polymers, such as PDMS or poly (ethylene glycol) (PGA) based chemicals [32]. Naturally derived polymers, in comparison to synthetic polymers, often exhibit favorable cell/material interactions [32-34].

Apart from allowing cells to grow in a more *in vivo* like topography, microfabrication techniques can also provide the means to define the chemical environment of the scaffold in the µm range. 2D chemical micropatterning allows a surface to be selectively coated with different chemicals, e.g. ECM molecules providing ligands for cell adhesion [32], in a wide range of geometries, sizes, ligand types and densities [15].

Fabrication methods for 2D chemical micropatterning include soft lithography methods such as microfluidic patterning, stencil-assisted patterning and micro-contact printing. In microfluidic patterning microchannels are used to flow the chemicals in specific patterns over the substrate. The microchannels are then removed and the patterned substrate can be used. In stencil-assisted patterning cells are seeded onto a substrate through the holes of the stencil. Once the stencil is removed it is possible to repeat the process and add another cell type.

In micro-contact printing a stamp, made from some soft material, is used to deposit the chemical onto the substrate [15].
By defining the chemical environment on the µm-scale cells can be affected and studied in several ways. Cell spreading can be restricted to certain areas. In this way, by varying the area coated with ECM molecules, endothelial cells can be stimulated either to spread or to undergo apoptosis [35]. Also, cell polarity in neurons, at single cell level, is possible to control using micro-patterned scaffolds [36]. The geometry [37] and size [38] of patterns have been shown to affect whether hMSCs (human mesenchymal stem cells) differentiate into adipocytes or osteoblasts.
A method which can be used both to define micro-topography and the chemical environment on the µm-scale is UV lithography. By selectively exposing a material to UV-light through a photomask it is possible to induce chemical reactions only in defined areas, on the µm-scale.

2. My contribution to the research field

The two papers I have contributed to show the possibilities of using a traditional, much used and well known method, UV lithography, on hydrogels which are biocompatible ECM-like materials.
I have demonstrated how UV lithography enables both selective cross-linking and attachment of chemical groups which promote cell adhesion to hyaluronic acid hydrogels. In this way cell culture scaffolds can be tailor made on the µm-scale and both provide a varying topography as well as a varying chemical environment to the cells of the *in vitro* model in the size range of individual cells.
The biocompatibility of the hyaluronic acid hydrogels, with either gelatin or RGD (Arginine-glycine-aspartic acid) peptides added to improve the material's ability to support cell adhesion, was investigated by using them as cell culture scaffolds for Caco-2 cells, a cell line used to model the epithelial cells of the gastro intestinal tract, and bEnd.3 cells which are used to model endothelial cells in the brain.
However, there are far more applications, as the UV lithography patterns both for selective cross-linking and attachment of chemicals, as well as which chemicals are attached, can be defined for each specific purpose.
Thus, a cell culture scaffold patterned with UV lithography provides a varying biochemical and biophysical environment which cannot be achieved in the traditional cell culture flasks or Petri dishes.

3. Materials

3.1 Hydrogels

Because many of the natural ECM constituents are hydrogels, this material type is of great interest also when designing new cell culture scaffolds, both for *in vitro* models and for tissue engineering [15]. Hydrogels are a class of materials consisting of three dimensional networks of polymers capable of retaining large amounts of water [39]. The polymers can be either natural or synthetic and the polymer chains are cross-linked through chemical crosslinking, i.e. covalent bonding, or physical crosslinking i.e. hydrogen bonding, van der Waals forces or physical entanglement [40]. Because of their capacity to retain large amounts of water hydrogels present a soft and rubbery consistency, much resembling that of living tissue [41].
Hydrogels can be found in a variety of everyday products such as soap, shampoo, toothpaste, hair gel, contact lenses [42] and many types of foods. Hydrogels can also be used for various biomedical applications including drug delivery and wound dressing [43]. Synthetically derived hydrogels include e.g. poly (ethylene glycol) (PEG), poly (vinyl alcohol) (PVA) and poly (2-hydroxy ethyl methacrylate) (pHEMA). Their advantage is that they can be synthesized in a controlled manner and thus show a low variability combined with a tight control of the structure of the finished polymer. However, synthetic hydrogels lack factors that promote cell behavior, and act mainly as a template to support cell function [33]. Naturally derived hydrogels have the advantage of possessing inherent bioactive properties [34], including integrin-binding sites and growth factors to a greater extent than synthetic polymers do [33]. Collagen and hyaluronic acid are both examples of naturally derived hydrogels found in the natural ECM.

3.1.1 Hyaluronic acid

Hyaluronic acid (HA) is a glycosaminoglycan and one of the primary components of the natural ECM [44]. It was previously mainly ex-

tracted from rooster combs but there are now microbial techniques where the hyaluronic acid is harvested from S*treptococci* bacteria via fermentation [45]. The HA repeating unit consists of glucuronic acid and N-acetyl-glucosamine and is depicted in Figure 2.

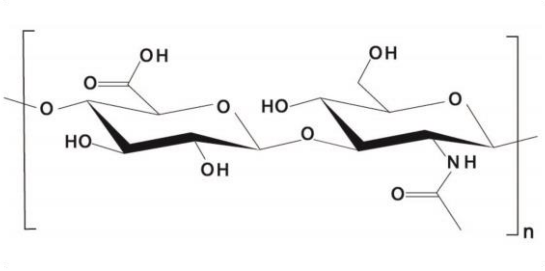

Figure 2 The HA repeating unit consisting of glucuronic acid and N-acetyl-glucosamine.

Along the backbone of the HA there are carboxylic acids and alcohols which allow for introduction of functional groups for crosslinking [46]. By crosslinking it is possible to form HA hydrogels. Many different cross-linking chemistries have been reported including homogenous reactions, in which the crosslinking agent is added directly into the HA solution, and heterogeneous methods where a HA "sponge" is immersed into a solution containing the crosslinking agent [47]. By choosing functional groups that require exposure to UV light, in combination with a photoinitiator forming free radicals to initiate the crosslinking reaction, it is possible to achieve a hydrogel which only crosslinks upon UV exposure and thus is possible to photo-pattern. Although hyaluronic acid is a natural hydrogel found in the ECM it does not support cell adhesion. However, other natural polymers, such as gelatin, which contain binding sites for integrin adhesion, can be incorporated within the hydrogel matrix [48]. Also, the same carboxylic acids and alcohols which allow for introduction of functional groups for cross-linking can be utilized to covalently link motifs such as the RGD peptide which can be recognized by cell surface adhesion molecules [44].

3.1.2 Gelatin

Gelatin is a hydrolyzed form of collagen which is the most abundant structural protein of the ECM [48]. It is extracted by boiling animal skins, bones, and tissue after alkali or acid pretreatment [49].
Gelatin is used to coat standard cell culture flasks and Petri dishes to promote cell adhesion for many different cell types [48]. The reason for this is that it contains several bioactive motifs, such as the RGD peptide [50].
Gelatin forms a thermoreversible gel due to the formation of micro-crystallites where the polymer chains meet. These microcrystallites function as physical crosslinks but are broken if the gelatin is heated above the melting point [51].

3.2 The RGD peptide

Arginine-glycine-aspartic acid, or RGD, is a tripeptide, composed of the three amino acids L-arginine (R), glycine (G), and L-aspartic acid (D). It plays an important role *in vivo* for the attachment between the ECM and cells. The RGD sequence is recognized by adhesion proteins, integrins, on the surface of the cells [52]. Many types of integrins recognize the RGD sequence and it thus promotes cell adhesion in many cell types.

4. Methods

4.1 Functionalization of hyaluronic acid with acrylamide

As previously mentioned, along the backbone of the HA there are carboxylic acids and alcohols which allow for introduction of functional groups for crosslinking [46]. Acrylamide can be covalently linked to the carboxylic group of the HA. By exposure to UV light, in the presence of a photoinitiator forming free radicals, the crosslinking reaction of HA functionalized with acrylamide is initiated, Figure 3.

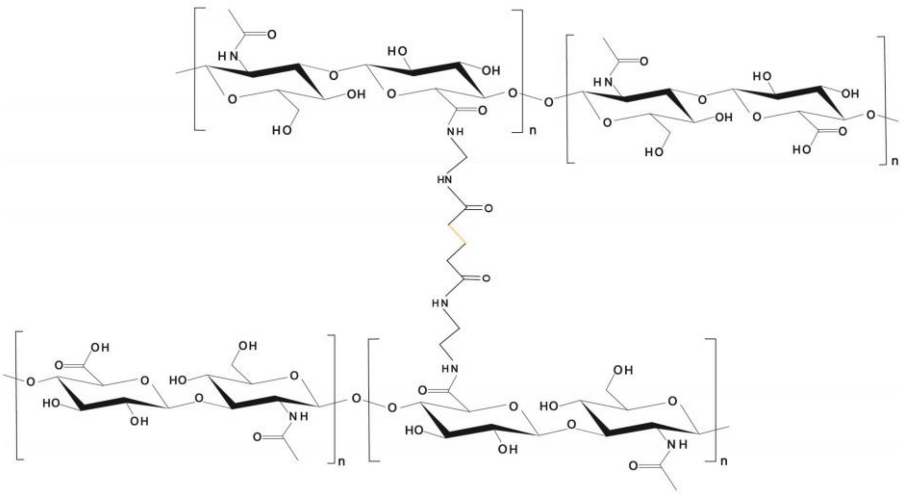

Figure 3The crosslinking of HA functionalized with acrylamide. The reaction is initiated by the exposure to UV light in the presence of a photoinitiator forming free radicals.

4.2 Photolithography

Photolithography is one of the most important techniques within the field of microfabrication. The photolithography process involves the

use of an optical image and a photosensitive film to produce a pattern on a substrate. It generally relies on the use of photosensitive polymers called photoresists [53].
Photoresists are divided into two sub-groups; negative and positive, Figure 4. Negative photoresists become less soluble when exposed to light whereas positive photoresists become more soluble. The process steps in photolithography involve the formation of a thin photoresist-film on top of the substrate, which is often done with spincoating. Typically the film thickness is between 0.5 µm and 2 µm [53].
The next step is exposure to light, usually at 365 nm wavelength (UV light), through a photomask that defines the desired pattern. The photomask is a plate, often in quartz-glass, with a layer of an opaque material, often chromium, which allows light to shine through in a specific pattern [53].
After this the next step is development, which involves immersing the substrate with the exposed photoresist into a chemical that dissolves either exposed photoresist (if using positive photoresists) or the non-exposed photoresist (if using negative photoresists) [53].

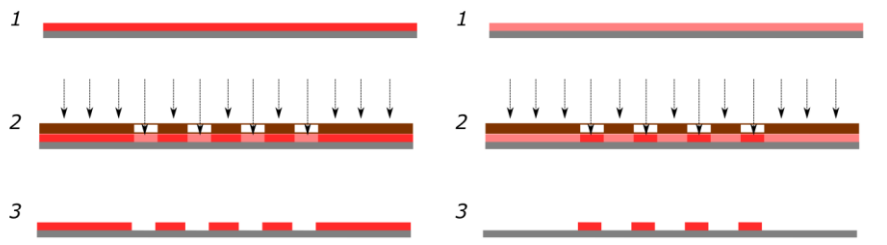

Figure 4 The steps of photolithography. (Left) positive photoresists, (Right) negative photoresists. The process includes (1) formation of a thin film of photoresist on top of the substrate, (2) exposure to light through a photomask and (3) development, removing the exposed part of the positive photoresist, or the non-exposed part of a negative photoresist.

The photolithography steps are then usually followed by other process steps such as dry- or wet etching or deposition of thin films [53].
One example of a photoresist is the above mentioned (1.3 Improved *in vitro* models, cell culture scaffolds) negative epoxy-based SU-8 photoresist [31]. It is often used as mold for e.g. PDMS casting.
The quality and the resolution of the pattern defined in the photolithography process are dependent on several factors. One is the thickness of the photoresist film. In a thicker film there will be more light scattering than in a thinner one and thus, if a high resolution pattern is

needed, a thinner photoresist film is needed. Also, the pattern defined usually cannot have a higher resolution than the photomask. Many photomask writers have a resolution limit in the range of 100 µm up to 1 µm.
The ultimate resolution limit though is the wavelength of the light [54]. Most photoresists are sensitive to light with wavelengths in the 300 nm to 500 nm and the most commonly used light source, the mercury vapor lamp, emits light from 310 nm to 440 nm [53]. If a higher resolution is needed, x-rays, which have a wavelength of 4 Å to 50 Å, can be utilized. Also, if moving from electromagnetic radiation, and to electron beam lithography, it is possible to define patterns in the sub-10 nm range. For this purpose, special electron sensitive resists are used [54].
Another important factor when it comes to the quality of the defined pattern is the cleanliness of the lab environment. As patterns are defined in the µm- or even nm-range any dust particle ending up on the substrate could prove fatal to the result. Because of this most photolithography processing is done within clean room facilities which offer environments with less than 350 particles with size >0.5 µm per cubic meter. This can be compared to the ~35 million particles per cubic meter being present in normal indoor air [53].

4.2.1 Selective crosslinking of hyaluronic acid-acrylamide using photolithography

As the cross-linking of HA-acrylamide is induced by exposure to UV light, it is possible to use photolithography to form patterns directly in the hydrogel Paper I.
There are, of course several other crosslinking chemistries which can be used to crosslink HA, and also a few ones which are photoinduced, such as methacrylated HA [55]. However, in this section the photolithography, or selective crosslinking, of HA-acrylamide will be described.
The crosslinking reaction of HA-acrylamide is initiated by activation of a photoinitiator which forms a free radical upon exposure to light of a specific wavelength, Figure 5. The photoinitiator Irgacure 2959 (2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone, Ciba Specialty Chemicals was used in Paper I.

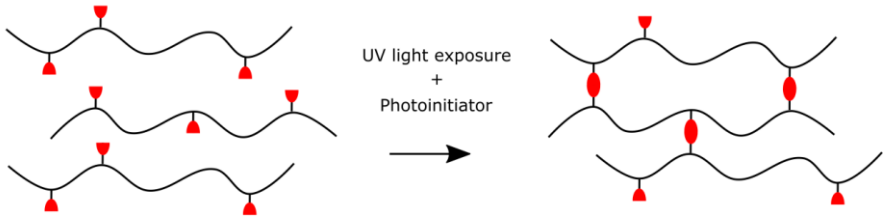

Figure 5 Crosslinking of HA-acrylamide. Upon exposure to UV light the photoinitiator forms free radicals which initiate the crosslinking reaction between the acrylamide groups (red).

The process for photolithography, or specific crosslinking, is similar to that of "normal" photolithography using negative photoresists, with a few exceptions, Figure 6. At first, the photoinitiator is dissolved in degassed water. After this, the HA-acrylamide derivative is dissolved in the same solution. As a neutral pH is desirable for cell culture, a base, such as sodium hydroxide, is added.
Next, a film of the solution is formed between a substrate and the photomask, using a spacer to define the distance and thus the film thickness. The hydrogel is then exposed to light through the photomask. The pattern is developed by immersion into water or phosphate buffered saline (PBS) and any non-crosslinked part of the hydrogel solution is washed off.

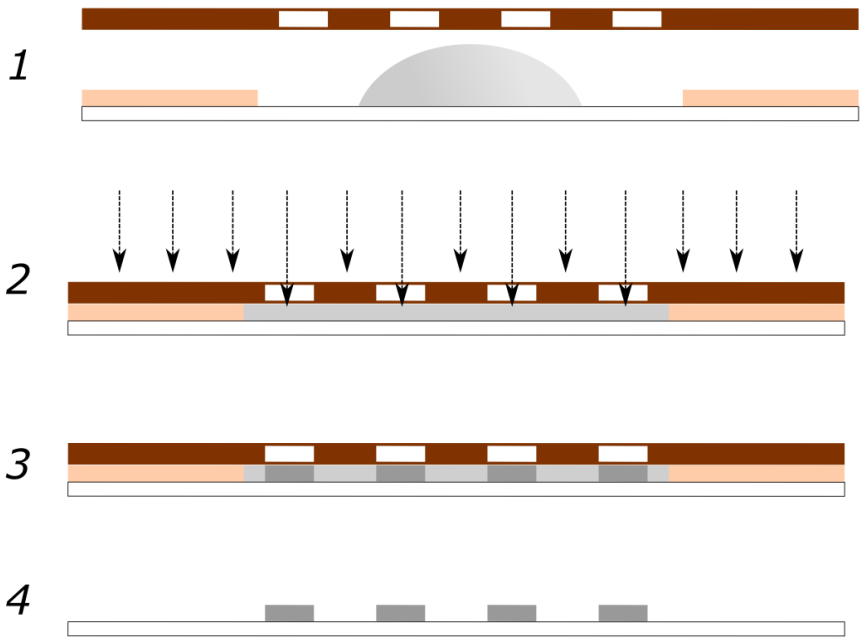

Figure 6 The steps of photolithography on HA-acrylamide. The process includes (1) formation of a thin film of photoresist between the substrate and the photomask with spacers defining the distance and thus the film thickness, (2) exposure to light through a photomask and (3) development in water or PBS, washing off any non-crosslinked part of the hydrogel solution.

The light dose and wavelength are of course vital parameters for the result of the photopatterning of HA-acrylamide.
The required wavelength depends on the photoinitiator used. A higher wavelength is favorable if cells are to be encapsulated within the hydrogel. Also, the light dose has an influence on the result. A too low dose will not be sufficient to crosslink the hydrogel whereas a too high dose will form a yellowed hydrogel.
The required light dose for crosslinking is also dependent on the photoinitiator concentration, the HA-acrylamide concentration and the degree of functionalization of the HA.
Just as for photolithography using photoresists the thickness of the film being exposed is of importance for the resolution. Whereas a typical photoresist thickness is in the single µm range the film thickness of the HA-acrylamide hydrogel used in Paper I was closer to 100 µm. For the intended application it was the topography that was of interest

and thus the thicker film was needed. Of course, as the film thickness was high there was a lot of light scattering taking place throughout the material, and the result was a continuous piece of hydrogel with the structures defined in the photomask protruding from the surface, Figure 7. The topography could however be defined in a size range of 5-10 µm which is smaller than most mammalian cells. A thinner hydrogel could probably enable a higher resolution and structures more resembling those in Figure 7 a. However one has to take into consideration that a hydrogel film in the µm range would dry extremely fast and some type of humidity chamber would be necessary during the patterning process.

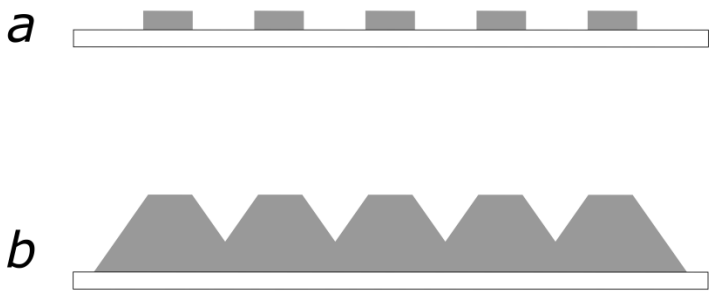

Figure 7 Photolithography result when using (a) thin polymer films and (b) thick polymer films.

In order to minimise the light scattering, and maximise the resolution it is important to have a short distance between the light source and the sample, both if working with thin or thick films.
Several studies have been performed on the viability of cells encapsulated within photo-crosslinked hydrogels. Many studies have found the Irgacure 2959 to be the best commercially available photoinitiator when it comes to low cytotoxicity [56]. The maximum absorption of Irgacure 2959 is found at ~270 nm [57]. However, as the absorption peak is quite broad also wavelengths up to 380 nm are possible to use [57]. A survival rate of ~95 % in human neonatal fibroblasts has been shown when encapsulated within PEG-diacrylate hydrogels crosslinked using Irgacure 2959 and exposed to light at 365 nm [58].
The UV light dose has been found to have an effect on cell viability when exposing cells to wavelengths in the UVA range (315-400 nm).

It has been found that doses in the range of 5-10 J/cm^2 cause minimal cell death if used together with low photoinitiator (Irgacure 2959) concentrations. In studies using higher doses, around 18 J/cm^2, indications of DNA damage were shown, and at even higher doses, 50 J/cm^2 the light caused severe damage to the cell membrane [59]. However it has to be noted that these studies all varied in cell type used, wavelength of the UV light, hydrogel chemistry and photoinitiator concentration. Thus, if performing photolithography on hydrogels containing cells (encapsulating cells) it is wise to test how these parameters affect the cell viability and behavior for each individual case. If seeding cells on top of the hydrogel however, the light exposure only has to be optimized for the patterning without consideration to the cells as they are added after the exposure step.

4.2.2 Specific patterning of RGD peptides in a hyaluronic acid-acrylamide matrix using photolithography

As previously mentioned the RGD peptide is recognized by adhesion proteins, integrins, on the surface of many cell types. Because of this, linking RGD peptides to the HA is an efficient way of promoting cell adhesion to the hydrogel. RGD peptides are available commercially and can be bought with different side groups and fluorescent tags. By using RGD peptides to which a thiol group has been added these can be covalently linked to the HA-acrylamide through a thiol-ene reaction between the thiol and the acrylamide.
The reaction between the thiol groups and the acrylamide is initiated by a photoinitiator forming free radicals upon exposure to light. Therefore it is possible to selectively add the RGD peptides to the HA by using a photomask during the exposure step.
In the first step of the process, the HA-acrylamide hydrogel is formed upon exposure to light. However, the light dose is chosen to be low enough to only make the photoinitiator form free radicals enough for crosslinking the hydrogel partially. Thus, there will be unreacted acrylamide groups left on the HA backbone which have not yet been linked to anything. After this, a solution containing RGDs is added on top of the hydrogel and upon exposure to a second dose of light, the thiol-ene bond is formed, Figure 8.

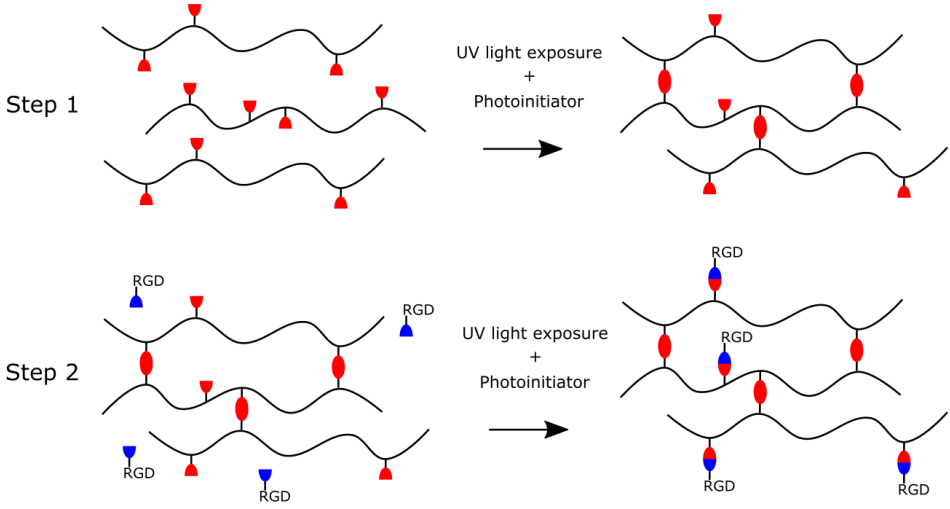

Figure 8 Crosslinking of HA-acrylamide and addition of RGD peptides. Upon exposure to UV light the photoinitiator forms free radicals which, in the first step, initiate the crosslinking reaction. During the second exposure step any acrylamide (red) which has not linked to another acrylamide group is free to react with the thiol groups (blue) attached to the RGD peptides, forming a thiol-ene bond.

The process for preparing the HA-acrylamide hydrogels with RGD patterns include forming a thin film of HA-acrylamide between a mold made from SU8 and silicon and a “lid” which is a glass cover slip, Figure 9. The first exposure step is done through the glass cover slip. After this, the mold and the glass are separated, leaving the hydrogel film on the glass cover slip. A droplet of a solution containing the RGD peptides is then placed on top of the hydrogel film. The photomask is placed on top and the second exposure step is performed. After the second exposure step the hydrogel is rinsed in PBS or water, washing off any RGD peptides which have not been attached to the surface of the HA-acrylamide hydrogel.

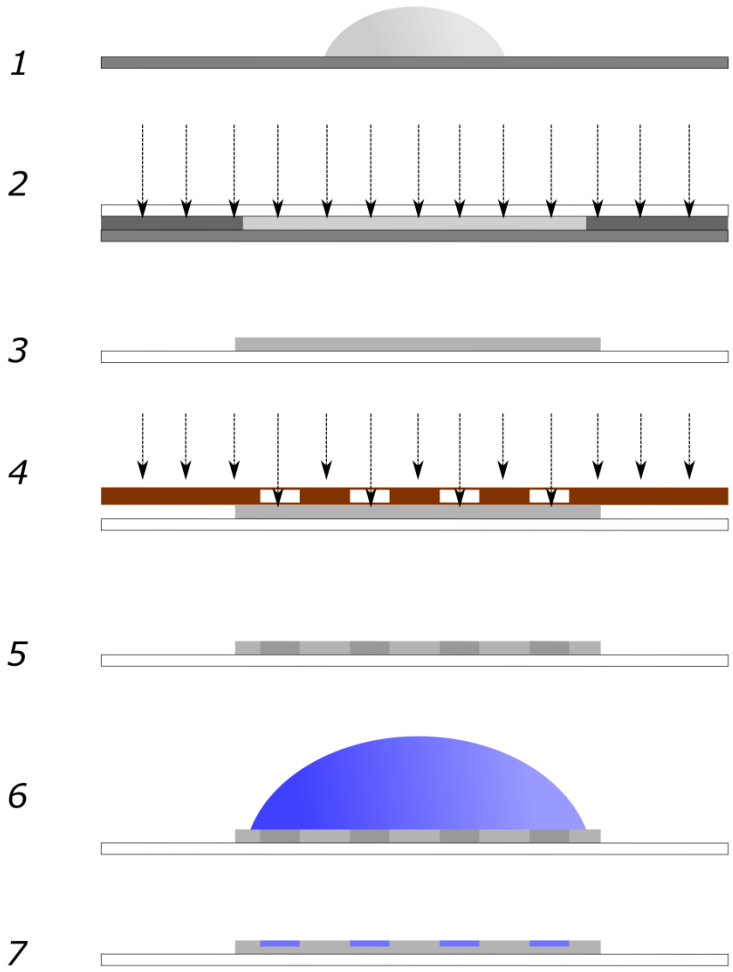

Figure 9 The steps of specific patterning of RGD peptides in a HA-acrylamide matrix. The process includes (1) adding a droplet of a HA-acrylamide solution onto a SU8/silicon mold and (2) exposing the HA-acrylamide to light through a glass cover slip forming a film of crosslinked hydrogel (3). In a second exposure step (4) a photomask is used to locally initiate the formation of free radicals in a pattern defined by the photomask (5). A droplet of a solution containing the RGD peptides (6) is added onto the hydrogel film and the RGD peptides are covalently linked to the HA-acrylamide. Any non-attached RGD peptides are washed off leaving a HA-acrylamide hydrogel with RGD peptides attached in a pattern (7).

If the RGDs have a fluorescent tag attached this allows for fluorescent imaging of the pattern.

4.3 Addition of gelatin into the hyaluronic acid matrix

In order to enhance the ability to support cell adhesion of the HA-acrylamide hydrogels it is possible to incorporate other natural hydrogels, such as gelatin which naturally contain cell adhesion motifs, into the hydrogel matrix. The gelatin is added to the HA-acrylamide solution before the crosslinking step. As the chemical crosslinks between the acrylamide have been formed during the light exposure step, and the hydrogel has been cooled below the melting point of gelatin, the result is a hydrogel network consisting of physically entangled HA-acrylamide and gelatin chains where the HA-acrylamide chains are chemically crosslinked to each other and the gelatin chains are physically crosslinked to each other, Figure 10. Hydrogels with more than one polymeric network interlaced with another is referred to as interpenetrating polymeric network (IPN) hydrogels [60].

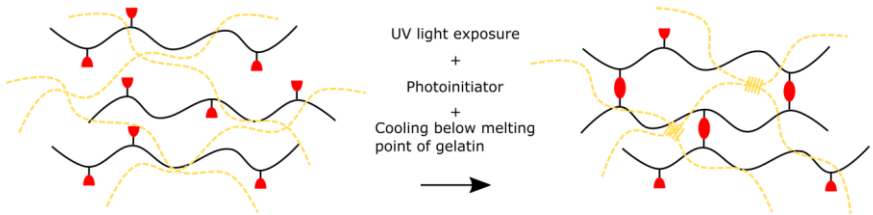

Figure 10 Crosslinking of HA-acrylamide and gelatin hydrogels. As the solution containing both HA-acrylamide and gelatin is subjected to light exposure and is cooled below the melting point of gelatin a polymer network consisting of chemically crosslinked HA-acrylamide (black/red) and physically crosslinked gelatin (yellow, dashed lines).

Because only the crosslinking of HA-acrylamide is sensitive to light it is only this crosslinking reaction which enables photopatterning (or selective crosslinking). Therefore the patterning process is sensitive to the amount of gelatin added. A too high gelatin to HA-acrylamide ratio will result in a hydrogel which is possible to crosslink, but the pattern will not be replicated successfully.

4.4 Surface treatment of glass cover slip substrates

In order to allow for handling of the patterned HA-acrylamide hydrogel films it is helpful to keep them on some type of substrate. A cover

slip glass is convenient as it is transparent and thus does not preclude imaging of the hydrogels.
However, if forming the hydrogel film directly on a non-treated cover slip glass the adhesion will be poor. This can cause problems especially if the hydrogel film is to be soaked in PBS or cell culture media as a floating hydrogel film is difficult both to seed cells on and to take images of. Therefore a surface treatment to increase the adhesion between the hydrogel and the substrate is required. Different silanes are much used for surface treatments as they covalently linked to hydroxyl groups of both silicon and glass [61]. By treating the surface of the cover slip glass with 3- mercaptopropyltrimethoxysilane, which provides thiol groups (also known as mercapto groups), the HA-acrylamide can be covalently linked to the surface of the cover slip glass. This is due to a thiol-ene reaction between the acrylamide groups of the HA-acrylamide and the thiol groups of the 3- mercaptopropyltrimethoxysilane, Figure 11. The reaction is initiated by exposure to UV-light and the formation of free radicals in the photoinitiator.

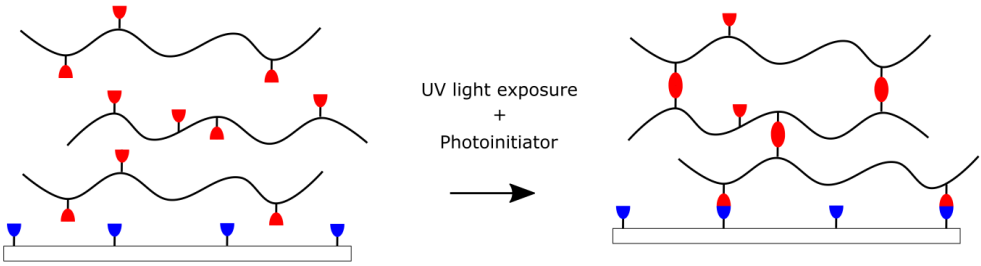

Figure 11 Covalent bonding between the HA-acrylamide hydrogel and surface treated cover slip glass. The cover slip glass has been treated with 3- mercaptopropyltrimethoxysilane leaving thiol groups (blue) on the surface with which the acrylamide (red) reacts in a thiol-ene reaction initiated by free radicals formed in the photoinitiator upon exposure to light.

During selective crosslinking of the HA-acrylamide and specific patterning of RGD peptides the covalent bond to the cover slip glass takes place during the crosslinking reactions, step 2 in Figure 6 and step 4 in Figure 9. This surface treatment allows for easier handling of the HA-acrylamide hydrogel films. Also, it does not have any negative effect on cell culture, and cells can even attach on the silanized glass surfaces.

4.5 Characterization

4.5.1 Imaging

When doing selective crosslinking of HA-acrylamide or specific patterning with RGD peptides imaging of the resulting patterns is an important step to evaluate the result. Imaging of hydrogels does however provide some challenges as the hydrogels are nearly transparent, dry if not kept in liquid and are not electrically.

When evaluating selective crosslinking of HA-acrylamide using light microscopy there are two alternatives to overcome these challenges. Dried samples can be used for imaging. The pattern is often to a large extent maintained even after the samples have been dried, however the features of the pattern are likely to have decreased in size. Another possibility is to use an inverted microscope, i.e. looking at the samples from below. This of course requires transparent substrates, such as a cover slip glass. The cover slip glass with the hydrogel on top is placed at the bottom of a well plate or a Petri dish and thus the sample can be kept in liquid. This also allows for imaging of cells growing on top the hydrogel.

The RGD peptides are commercially available with fluorescent tags which enable visualization of patterns using fluorescence microscopy. However, during the light exposure step and also if the samples are sterilized using UV light it has to be taken into consideration that a too high light dose might bleach the fluorescent tag.

To achieve more high resolution images of the selectively crosslinked hydrogel patterns, or the structure of the hydrogel itself on a detailed level, Scanning Electron Microscopy (SEM) is often required. The SEM technique only allows for imaging of electrically conductive samples that can withstand high vacuum and thus the hydrogel samples need to be dried and coated with a thin (only a few nm is required) conductive metal layer before imaging [62].

Also Environmental Scanning Electron Microscope (ESEM) techniques are available. Water vapor can be introduced into the ESEM sample chamber, thus keeping it a certain pressure. By simultaneously cooling the samples with a peltier element the chamber can be kept at a high relative humidity without introducing as many water molecules as would otherwise be required and thus the samples are not dried out. The water vapor does however cause scattering of the electron beam and the resolution is often not as high as for a conventional SEM [62].

The topography of the samples is also of interest. Optical techniques such as Vertical Scanning Interferometry (VSI) can be used on dry samples. However the topography of a dry sample is very different from that of a non-dried sample. Using special additional equipment that can compensate for the optical path if scanning a sample in liquid can enable imaging of non-dried samples. Non optic methods, such as stylus profilometers could be possible to use but often require a too high pressure resulting in the stylus rather scratching the surface of the hydrogel than scanning it. In Atomic Force Microscopy (AFM) the force between the sample and the probe is extremely weak and there is thus no issue with the probe scratching the surface of the hydrogel. However the samples would either have to be dry or special additional equipment allowing for scanning in liquid is required. The AFM provides higher resolution than the VSI, but cannot scan over as large surfaces or height differences [63, 64].

4.5.2 Release of gelatin from a hyaluronic acid-acrylamide matrix

When incorporating gelatin into the HA-acrylamide matrix it is of course of importance to know how much of the original amount of gelatin is maintained within the matrix under conditions resembling those of cell culture, i.e. in liquid media which is exchanged every two to three days. This is especially important as the gelatin content has a great influence on the hydrogel's ability to support cell attachment. By taking aliquots from the liquid into which the HA-acrylamide and gelatin hydrogels have been immersed the release of gelatin can be monitored over several days. The gelatin concentration of the aliquots is then easily measured using a protein quantification assay such as the Pierce BCA Protein Assay. In the Pierce BCA Protein Assay the proteins in the solution cause the reduction of copper ions, from Cu^{+2} to Cu^{+1}. The reduced copper ions then react with the bicinchoninic acid (BCA) forming a complex with a strong absorbance peak at 562 nm.
Similar assays are available also for detecting the presence of RGD peptides in solutions and release studies can thus be performed also for the hydrogels with RGD peptide patterns.

4.5.3 Swelling

The most remarkable property of hydrogels is their ability to retain large amounts of water. During the swelling process the polar hydrophilic groups of the hydrogel matrix are hydrated. After this, water also interacts with exposed hydrophobic groups. The osmotic driving force, which would otherwise lead to dilution of the hydrogel, is resisted by the physical or chemical crosslinks of the hydrogel matrix and additional water is absorbed. After this, an equilibrium state is reached [39]. The equilibrium swelling is often defined as the hydrogel's weight at equilibrium divided by its dry weight [65]. However, in our work, as we were interested in knowing how the swelling affects the pattern features of the hydrogels we choose to define the equilibrium swelling as the hydrogel's weight at equilibrium divided by its weight just after crosslinking.
As a swelling equilibrium is reached the amount of swelling that occurs after crosslinking can be accounted for when designing the pattern for selective crosslinking of HA-acrylamide.
The swelling of the hydrogels is most easily measured by immersing the hydrogel samples in liquid just after crosslinking and then weighing the samples to record their weight change over time.

4.5.4 Cell culture

In order to evaluate the suitability of the selectively crosslinked HA-acrylamide with gelatin incorporated into the matrix, and the HA-acrylamide with specifically patterned RGD peptides, as cell culture scaffolds, *in vitro* studies are performed.
The hydrogels, resting on their cover slip glasses, are put at the bottom of a wellplate and cell culture media is added. Cells are seeded on top of the hydrogels and the wellplates containing the hydrogels are put in an incubator. Because the wellplate, substrate and hydrogel are basically transparent the cells can be monitored using an inverted microscope.
The Caco-2 cell line was established from human adenocarcinoma during the 1970's. The Caco-2 cell line expresses several morphological and biochemical characteristics of small intestinal enterocytes including monolayer growth, polarized morphology with microvilli on one side, tight junctions between adjacent and enzymatic activity [66]. Because of this the Caco-2 cell line is routinely cultivated for studies of transports of drugs through the intestinal epithelium [67].

The bEnd.3 cell line is an endothelial brain cell line harvested from a mouse cerebral cortex. Cerebral endothelial cells are the structural basis of the BBB. The BBB restricts molecules and immune cells from entering the nervous system from the blood stream [68] and its effective barrier function is a result of the cerebral endothelial cells being tightly connected in tight junctions. The bend.3 cell line is thus an important tool to study how medicines, e.g. to treat Alzheimers disease, that can reach the nervous system can be developed [23].
Qualitative information about the viability of the cells on top of the hydrogels is most easily obtained by staining the cells with so called live/dead stains. The calcein-am/ propidium iodide assay is one example. The calcein-am (acetoxymethyl ester of calcein) can only penetrate the cell membrane of live cells, whereas the propidium iodide only can penetrate that of dead cells. As the two chemicals are excited and emit light at different wavelengths, the live and dead cells can be distinguished from each other [69]. To obtain quantitative data on cell viability, assays such as the alamar blue assay can be used. The weakly fluorescent resazurin is in viable cells reduced to resorufin, which is highly fluorescent. By measuring the intensity of the fluorescence signal, and comparing it to a standard curve with known cell concentrations, the amount of cells in a sample can be determined.
As tight junctions are important health markers of both intestinal epithelium and cerebral endothelium a good *in vitro* model of these two tissues should support the formation and quantification of the tight junctions. There are two main methods to evaluate the presence of tight junctions. These include fluorescent staining of tight junction proteins, including occludin and claudin, and transendothelial electrical resistance (TEER) that measures the electrical resistance over a cell layer on a cell culture membrane [23].

5. Summary of results

Paper I shows the selective crosslinking of the HA-acrylamide acid using UV lithography to spatially pattern the material into cell culture scaffolds. For improved ability to support cell adhesion, gelatin was added into the hydrogel matrix and physically trapped within the HA-acrylamide polymer network. The resolution limit was evaluated and the hydrogels were characterized with concern to their ability to retain the gelatin within the hydrogel matrix, swelling characteristics and their ability to support cell culture.

A pattern resolution of 5-10 µm in 100 µm thin hyaluronic acid films was shown. Also larger structures, in the mm range, could be defined within the hydrogel. The pattern features were not free standing, but rather part of a continuous piece of hydrogel as illustrated above in Figure 7b. The hydrogel retained about 50% of the added gelatin when kept in liquid for 9 days, and swelled 7 ± 5 % compared to the initial weight just after crosslinking, during this time.

Caco-2 cells were cultured on the selectively crosslinked HA-acrylamide and gelatin hydrogels. The cells were to a higher extent growing on the larger hydrogel structures (>160µm) than on the smaller parts (40 – 120 µm) of the pattern and in the larger structures the cell layers were nearly confluent after 5 days of culture. We speculate that this may be either due to the cells preferring larger structures to those in the size range of individual cells, as they for smaller patterns may recognize the shape of the features. It could also be that the cells did not adhere to the hydrogel directly after seeding, but the cells seen on the hydrogel are rather the ones which have migrated from the surrounding area (the bottom of the well-plate and the cover slip). It should be noted that the smaller pattern features were all located in the middle of the patterned area, and the larger structures were found at the edges. A combination of the two explanations is also possible. The features of the pattern furthest away from the edges which still had cells growing on them were in the size 160 µm. The features next to

the 160 µm ones had a size of 60 µm and on these no cells were found. It is thus a possibility that the cells migrated from the edges of the hydrogel towards the patterned area, but could not go any further as they recognized the smaller pattern features.

Paper II shows the specific patterning of RGD peptides in an HA-acrylamide hydrogel network. This was done by using RGD peptides connected to thiol groups which could link to any non-reacted acrylamide groups left on the HA after the hydrogel had been crosslinked. Squares, containing the RGD peptide, with sizes 100 µm by 100 µm and 200 µm by 200 µm were successfully defined and could be visualized as the RGD peptides used contained the 5-FAM fluorophore. Cells from the bEnd.3 cell line were seeded on the hydrogels patterned with 200 µm by 200 µm squares and it was found that the cells recognized the areas where the RGD peptides had been immobilized and selectively adhered to these areas.

6. Outlook

For future work there are many exciting things that may be explored. First of all it would be interesting to combine selective crosslinking of HA-acrylamide and specific patterning of RGD peptides within the hydrogel. This could be a promising technique to guide cells into growing in specific areas of defined topographies. Further studies should be performed to rule out if the size of the pattern features defined with selective crosslinking does have an effect on the cells willingness to adhere to the material. If there is such an effect it should of course be determined where the size-limit is. By culturing cells also on RGD peptide patterns with features with large variations in size it could be demonstrated if there is also such a size-limit for this method.

If, however, the case was that cells not adhering to the material directly after seeding there are different alternatives to enhance the materials ability to support cell adhesion. By incorporating gelatin with functional groups allowing for chemical crosslinking it should be possible to form a hydrogel with a larger capability of retaining the gelatin within the matrix, and thus providing more cell adhesion sites. The optimal functionalization would be one that allows for crosslinking induced by light as this would much facilitate the patterning process. Of course also the RGD peptides could be added to the HA-acrylamide solution before crosslinking, providing a hydrogel with uniformly distributed cell adhesion sites. Using a collimated light source or preparing thinner hydrogel films are possible approaches for reaching free standing hydrogel patterns.

In order to further characterize the biological features of the patterned materials the differentiation and formation of tight junctions between adjacent cells should be evaluated for different topographies. Also, the possibility to grow cells inside the hydrogel is of great interest. Co-cultures of e.g. Caco-2 cells and mucus producing cell lines for *in vitro* models of the gastro intestinal tract or endothelial cells and as-

trocytes in the case of the BBB could perhaps be interesting to further efforts on providing more *in vivo* like *in vitro* models.
Finally, combining the patterned hydrogels with microfluidic chips, possibly letting the hydrogels serve as the cell support within a microfluidic channel, gives the possibility to build an *in vitro* model with both an ECM like cell culture scaffold and physiological shear stress and flow.

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