Mestrado Integrado em Engenharia Química

Investigating Cellular Response to Engineered Hydrogel Substrates

Tese de Mestrado

Projecto de Desenvolvimento em Instituição Estrangeira

Filipa Meireles Maia

University of Maryland Baltimore County

Department of Chemical Engineering

Adviser: **Jennie B. Leach**

July 2008

Acknowledgments

All the work conducted during this Project would not have been possible without the helps of many people.

First of all, I would like to thank Dr. Jennie B. Leach, for all the help, suggestions and comments to the project during these 5 months.

I would also like to thank the PhD student Andreia Ribeiro for all her help, training and suggestions and for always being willing to contribute to the work.

Thank you to all the other students from Dr. Leach's lab, Miguel Acosta, Sylvia Petrova and Robert Reeves, for training me with different procedures and equipments in the laboratory.

Another thank you goes to Dr. Binil Starly from the University of Oklahoma, School of Industrial Engineering, and to his PhD student Ozlem Yasar, for working on the micropatterns and shipping them to us, and for being willing to work on all the problems we came across along the way.

Resumo

O estudo de interacções entre células em sistemas de co-cultivo tem-se revelado difícil de manipular com precisão em trabalhos anteriores de engenharia de tecidos. No entanto, a tecnologia de microfabricação tem evoluído e técnicas de estereolitografia podem ser usadas como ferramentas no controlo de interacções entre células de interesse.

Estudos anteriores com mais do que um tipo de células cultivadas em micropadrões basearam-se na selectividade de tipos diferentes de células aderirem a diferentes regiões bioactivas do material. No entanto, estes estudos podem apresentar algumas limitações no estudo da interacção entre dois tipos de neurónios, por exemplo. A principal ideia inovadora que está na base deste projecto é o *design* de materiais selectivamente degradáveis onde quaisquer dois tipos de células possam ser cultivadas para o estudo das interacções entre células *in vitro*. Usando micro-estereolitografia de laser (μSL) foram desenvolvidos micropadrões milti-componente bem definidos, constituídos por polietileno glicol – dimetacrilato (PEG-DM) e carboximetilcelulose – aminoetil metacrilato (CMC-MA), sobre superfícies de vidro quimicamente modificadas. Uma vez que ambos os polímeros são resistentes à adrosção de células e proteínas, o primeiro tipo de células é obrigado a aderir à superfície livre do vidro. A degradação do padrão de CMC-MA através da acção enzimática da celulase irá dar origem a novas regiões livres no material onde um segundo tipo de células pode ser cultivado. Através desta técnica é possível controlar o contacto e o espaçamento entre os dois tipos de células a um nível micrométrico. Este modelo pode ainda ser utilizado como uma ferramenta para o estudo e a clarificação dos mecanismos de transmissão de sinais intercelulares.

Tanto a partir de estudos de degradação da CMC-MA sob o efeito da celulase, como de estudos da viabilidade de 3T3 fibroblastos sob a mesma enzima, foi possível concluir que a concentração óptima de enzima para a degradação da CMC-MA nos micropadrões é de 0.5 U/mL. Foi também provado que o PEG-DM não é degradado por esta enzima e a compatibilidade destes matérias com as células foi comprovada. A experiencia completa com o cultivo dos 3T3 fibroblastos, a degradação do padrão de CMC-MA e o cultivo das células PC12 foi realizada em macro padrões de PEG-DM/CMC-MA e assim que a mesma experiencia seja realizada nos micropadrões, a aplicabilidade desta técnica poderá ser comprovada.

Palavras-chave: Engenharia de tecidos; Micro padrões; Co-culturas; Modificação de superfícies de vidro; Polietileno glicol; Carboximetilcelulose.

Abstract

The study of cell-cell interactions in co-culture systems has revealed difficult to manipulate accurately in previous tissue engineering reports. However, microfabrication technology has evolved and stereolithography techniques can be used as tools to control cell-cell interactions of interest.

Previous studies with micropatterned co-cultures were based in the selectivity of two distinct cell types to adhere to different bioactive regions of the material. However, those studies may present some challenges when studying the interaction between two types of neurons, for example. The main innovative idea behind this project is to design selectively degradable materials where *any* two cell types can be cultured for the study of cell-cell interactions *in vitro*. Using laser micro-stereolithography (μSL) defined multi-component micropatterned architectures of poly(ethylene glycol) – dimethacrylate (PEG-DM) and carboxymethylcellulose - aminoethyl methacrylate (CMC-MA) were developed on top of chemically modified glass surfaces. Since both polymer surfaces are resistant to cell and protein adsorption, the first cell type is forced to adhere to the free glass spaces. The degradation of the CMC-MA patterns by the enzymatic action of cellulase will provide new adhesive regions in the material where a second cell type can be seeded. With this technique it is possible to control cell-cell contact and spacing between two different cell types at the microscale. This model can be further used as a tool for the study and clarification of intercellular signalling mechanisms.

From degradation studies with CMC-MA under cellulase as from cell viability studies of 3T3 fibroblasts under the same enzyme, it was possible to conclude that the optimal concentration of enzyme for the degradation of CMC-MA in the micropatterns is 0.5 U/mL. It has been proven that PEG-DM is not degraded by this enzyme and the cell compatibility of the materials was confirmed. The full experiment with the culture of 3T3 fibroblasts, the degradation of the CMC-MA pattern and the culture of PC12 cells was conducted on macro PEG-DM/CMC-MA patterns and once the same experiment is conducted on the micropatterns, the applicability of the technique can be proven.

Key-words: Tissue engineering; Micropatterning; Co-cultures; Glass surface modification; Polyethylene glycol; Carboxymethyl cellulose.

Index

Index i

Glossary iii

1 Introduction 1

1.1 Background and Presentation of the Project 1

1.1.1 Tissue Engineering 1

1.1.2 Scaffold Design 1

1.1.3 Hydrogels 2

1.1.4 Cell-cell interaction 4

1.1.5 Selectively Degradable Hydrogels for the Study of Cell Interactions in Co-coltures 4

1.2 Contributions of the Work 6

1.3 Organization of Thesis 7

2 State of the Art 9

3 Technical Description 12

3.1 CMC-MA synthesis 12

3.2 CMC-MA conjugation with 5-amimoeosin 13

3.3 Degradation of CMC-MA under cellulase 14

3.3.1 Degradation of macro CMC-MA hydrogels under cellulase 14

3.3.2 Degradation of CMC-MA thin layers under cellulase 16

3.4 Degradation of PEG under cellulase 17

3.5 Cell viability under cellulase 18

3.6 Modification of glass coverslips 18

3.7 Micropatterns 21

3.8 CMC-MA/PEG-DM patterns 21

4 Results and Discussion 24

4.1 Degradation of CMC-MA under cellulase 24

4.1.1 Degradation of macro CMC-MA hydrogels under cellulase 24

4.1.2 Degradation of CMC-MA thin layers under cellulase 29

4.2 Degradation of PEG under cellulase 34

4.3 Cell viability under cellulase 35

4.4 Modification of glass coverslips 37

4.5 Micropatterns 40

4.6 CMC-MA/PEG-DM patterns 42

5 Conclusions 46

6 Evaluation of work conducted 47

6.1 Accomplished Objectives 47

6.2 Other work conducted 47

6.3 Limitations and Future Work 48

6.4 Final comment 48

References 49

Appendix 1 Degradation of CMC-MA thin layers under cellulase: images 52

Appendix 2 Neural Stem Cells Adhesion and Differentiation in Polyacrylamide Gels 60

Polyacrylamide hydrogels 61

Polyacrylamide hydrogels cultured with DRG's 62

Polyacrylamide hydrogels cultured with NSC 63

Appendix 3 Cell culture and staining 66

Appendix 4 Additional information 69

Glossary

Listo of acronyms

3D	Three-dimensional
µSL	Micro-stereolithography
AEM	Aminoethyl methacrylate
APS	Ammonium persulfate
CAD	Computer aid design
CMC	Carboxymethylcellulose
CMC-MA	Carboxymethylcellulose - aminoethyl methacrylate
DAPI	4',6-diamidino-2-phenylindole
$DiOC_{18}$	3,3'-dioctadecyloxacarbocyanine
DMD	Digital micro-mirror device
ECM	Extracellular matrix
EDC	*N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide
PBS	Phosphate buffer saline
PDMS	Polydimethylsiloxane
PEG	Poly(ethylene glycol)
PEG-DA	Poly(ethylene glycol) – diacrylate
PEG-DM	Poly(ethylene glycol) – dimethacrylate
PI	Propidium iodide
TEMED	Tetramethylethylenediamine

1 Introduction

1.1 Background and Presentation of the Project

1.1.1 Tissue Engineering

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue or organ function. It seeks to fabricate living replacement parts for the body (Bell, 2000).

Efforts are being made, and new achievements have been reached by combining several areas, like cell biology, material science, reactor engineering, and clinical research, to create environments that provide the development of new tissues whose properties more closely match their native counterparts. The goal of this approach is not to reproduce all the complexities involved in development, but rather to promote an environment which allows the native capacity of cells to integrate, differentiate and develop new tissues (Lavik and Langer, 2004).

1.1.2 Scaffold Design

The basic principle of tissue engineering is to couple the appropriate cells with a material under conditions which lead to tissue formation. The nature of the material and its physical and chemical properties are critical to creating the desired conditions for tissue formation (Lavik and Langer, 2004).

Numerous strategies currently used to engineer tissues depend on employing a material scaffold. These scaffolds are used as a synthetic extracellular matrix (ECM) to organize cells into a three-dimensional (3D) architecture and to present stimuli, which direct the growth and formation of a desired tissue (Yang et al., 2001).

The uses of both natural and synthetic polymers for tissue engineering have been under study. Some natural materials, like collagen, Matrigel and alginates have been used as scaffolds (Lavik and Langer, 2004). Since these materials compose the naturally occurring extracellular matrix, it makes sense to use them as the basis for engineered tissues. Particularly, collagen has successfully been used as a scaffold for skin repair (Pomahac et al., 1998) and several scaffolds based on it are currently available for clinical use. This material has also been used for nerve repair and bladder engineering (Lavik and Langer, 2004). Matrigel, which is a gel composed of basement membrane proteins, is also commercially available and has been used

for several purposes: to culture a wide variety of cell types, in spinal cord repair and in vascular networks (Lavik and Langer, 2004). Alginates have been used for several tissue engineering studies, including cartilage and cardiac tissues.

Though natural polymers have proved to be useful, there are certain limitations and concerns regarding their use. First, it is not easy to control their mechanical properties and degradation rates over a wide range (Lee et al., 2001). Second, it is possible that the naturally derived materials may cause a serious immune response or harbor microbes or viruses (Schmidt and Baier, 2000).

On the other hand, it is possible to tailor synthetic polymers to have a much wider range of mechanical and chemical properties than their natural counterparts. They also solve the problem regarding immunogenicity. However, biocompatibility becomes an issue (Lavik and Langer, 2004).

For the purpose of biomaterials, synthetic polymers may be classified as non-degradable and degradable polymers. Even though the non-degradable polymers can be produced with an extremely wide range of well controlled properties, their permanence raises a concern regarding their long-term effects, especially regarding scarring and inflammatory response. For that reason, there has been a great deal of research into the development of degradable synthetic polymers which would, in theory, have all the properties of their non-degradable counterparts, but also avoid the long-term issues by degrading to metabolizable components (Lavik and Langer, 2004).

1.1.3 Hydrogels

Polymer scaffolds have many different functions in the field of tissue engineering. They can be applied as space filling agents, as delivery vehicles for bioactive molecules, and as 3D structures that organize cells and present stimuli to direct the formation of a desired tissue. Much of the success of scaffolds in these roles is based on finding an appropriate material to address the critical physical, mass transport, and biological design variables inherent to each application (Drury and Mooney, 2003).

A variety a hydrogels, a class of highly hydrated polymer materials (water content ≥ 30% by weight), are being employed as scaffold materials (Park and Lakes, 1992). They are composed of hydrophilic polymer chains, which are either synthetic or natural in origin.

Hydrogels are an appealing scaffold material because of their structural resemblance to the extracellular matrix of many tissues, their general high biocompatibility, they can often be processed under relatively mild conditions, may be delivered in a minimally invasive manner, and their ability to be injected as a liquid which gels in situ (Drury and Mooney, 2003).

Therefore, hydrogels have been used as scaffold materials for drug and growth factor delivery, engineering tissue replacements, and a variety of other applications (Drury and Mooney, 2003).

The structural integrity of hydrogels depends on cross-links formed between polymer chains via various chemical bonds and physical interactions (Drury and Mooney, 2003). Hydrogels can either be chemically or physically cross-linked water-soluble polymers, and they may be either degradable or non-degradable, depending on their chemistry (Lavik and Langer, 2004).

Chemically cross-linked hydrogels can be polymerized in situ using either a chemical initiator (Stammen et al., 2001) or a photoinitiator (Nguyen and West, 2002). The photoinitiated hydrogels use initiators that only become active when they are exposed to light of a particular wavelength. The advantage of this process over standard initiation schemes is that the hydrogel gelation may be closely controlled: it is possible to inject the ungelled solution which is then gelled in an actively controlled manner (Lavik and Langer, 2004). Furthermore, photoinitiated hydrogels have been shown to be able to be injected with cells with high cell survival (Elissef et al., 1999; Mann et al., 2001) and used to deliver growth factors (Elissef et al., 2001).

A variety of synthetic and naturally derived materials may be used to form hydrogels for tissue engineering scaffolds. Synthetic hydrogels are appealing because their chemistry and properties are controllable and reproducible. Naturally derived hydrogel forming polymers have frequently been used in tissue engineering applications because they are either components of or have macromolecular properties similar to the natural ECM (Drury and Mooney, 2003).

Selection or synthesis of the appropriate hydrogel scaffold materials is determined by the physical properties (mechanics, degradation, gel formation), the mass transport properties (diffusion), and the biological properties (cell adhesion and signaling) required by each specific application (Drury and Mooney, 2003).

The biggest challenge with hydrogels has been to obtain a wide range of mechanical properties and to be able to produce controlled pore architectures (Lavik and Langer, 2004). Photopolymerization combined with photolithography is being investigated as a means to obtain distinct architectures (Liu and Bathia, 2002).

Once a specific material has been chosen for a scaffold-supporting structure, the material must be processed into a structure with the suitable architecture to support cell growth and tissue formation (Lavik and Langer, 2004).

Pore structures of hydrogels are generally defined by their cross-link densities and locations. The combination of photolithography and photopolymerizable hydrogels has been pursued to create patterned controlled pore architectures (Liu and Bathia, 2002).

1.1.4 Cell-cell interaction

The repair or replacement of damaged tissues using in vitro strategies has focused on manipulation of the cell environment by modulating of cell-ECM interactions, cell-cell interactions, or soluble stimuli. Even though many of these environmental influences are easily controlled using macroscopic techniques, in co-culture systems with two or more cell types, cell-cell interactions have been difficult to manipulate precisely using similar methods. Although microfabrication has been widely used for the spatial control of cells in culture, these methods have only recently been adapted to the simultaneous co-cultivation of more than one cell type (Bhatia et al., 1997). Microfabrication technology has evolved and stereolithography techniques can be used as tools to control cell-cell interactions of interest (Bhatia and Chen, 1999).

1.1.5 Selectively Degradable Hydrogels for the Study of Cell Interactions in Co-coltures

Previous studies with micropatterned co-cultures were based in the selectivity of two distinct cell types to adhere to different bioactive regions of the material (Bhatia et al., 1997; Hui and Bhatia, 2006). However, those studies may present some challenges if we desire to study the interaction between two types of neurons, for example. The main innovative idea behind this project is to design selectively degradable materials where *any* two cell types can be cultured for the study of cell-cell interactions *in vitro*.

Using laser micro-stereolithography (μSL) defined multi-component micropatterned architectures of poly(ethylene glycol) – dimethacrylate (PEG-DM) and carboxymethylcellulose - aminoethyl methacrylate (CMC-MA) were developed on top of chemically modified glass surfaces. These polymers were chosen because they are both biocompatible and CMC-MA is degradable. Since both polymer surfaces are resistant to cell and protein adsorption (Bhatia and Chen, 1999), the first cell type is forced to adhere to the free glass spaces. The degradation of the CMC-MA patterns by the enzymatic action of cellulase will provide new adhesive regions in the material where a second cell type can be seeded. In this technique it is possible to control cell-cell contact and spacing between two different cell types at the microscale. This model can be further used as a tool for the study and clarification of intercellular signalling mechanisms.

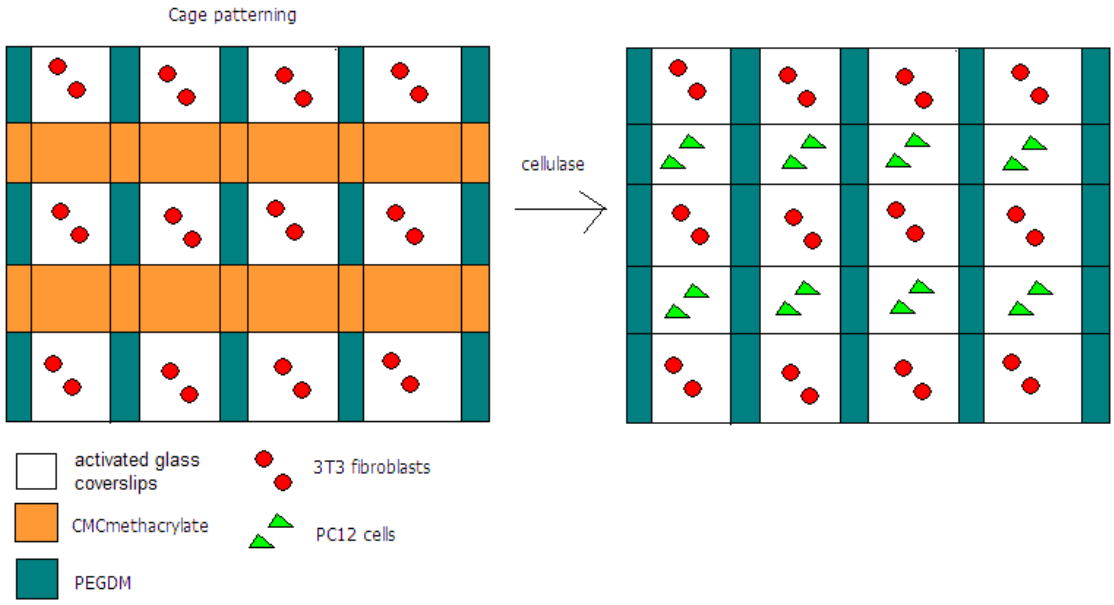

Figure 1. Schematic representation of the micropatterns and the cells.

The material synthesis and characterization and the cell culture studies were done in Dr. Jennie Leach's laboratory. The micropatterns were produced by a collaborator from the University of Oklahoma, School of Industrial Engineering, Dr. Binil Starly.

Figure 1 shows the patterns that Dr. Starly is currently working on, as well as a simple schematic of the procedure: culture 3T3 fibroblasts, degrade CMC-MA patterns, culture PC12 cells.

This technique will allow controlling cell-cell contact and spacing between two different cell types at the microscale.

The main goal is to study and improve this technique. If it can be shown that it is possible to study the cell-cell interaction between any two types of cells, further biological studies may be done with its application.

A future biomedical application of this technique would involve the study of neuromuscular transmission. For that purpose, a different pattern would be constructed, as the one shown in Figure 2, using the same technique. With this model, it would be possible to build a defined gap between the neurons and the muscle cells, simulating the synaptic cleft (the space between the axon terminal of the nerve cell in the spinal cord and the receptors on the muscle cell) and mechanisms regulating how the neurotransmitters released from the axon terminal reach the neurotransmitters on the muscle cell can be investigated.

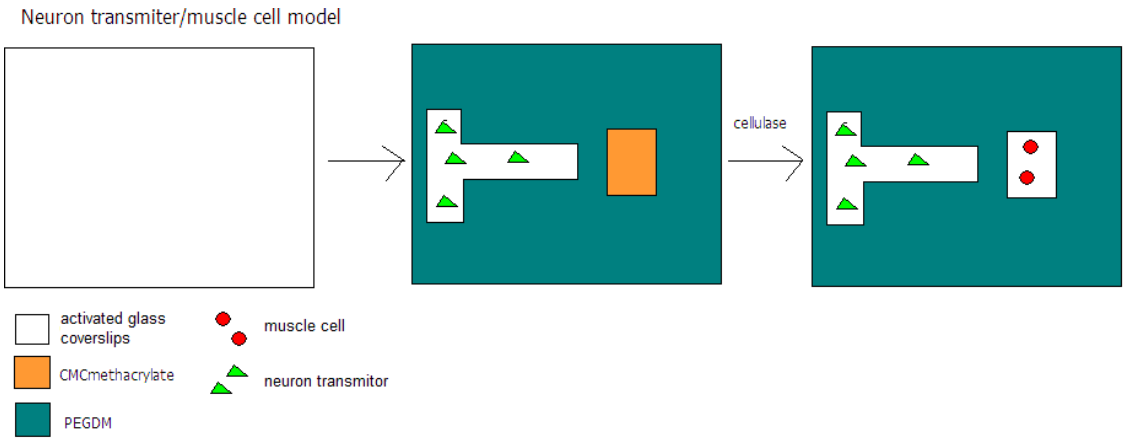

Figure 2. Schematic representation of the micropatterns needed for the study of the interaction between a neuron transmitter and a muscle cell.

The main objectives of this project are:

- Determine the optimal concentration of cellulase for the degradation of the CMC-MA pattern, through degradation studies of CMC-MA and cell viability studies of 3T3 fibroblasts;
- Prove that PEG-DM is not degraded by cellulase;
- Verify the cell compatibility of the micropatterns;
- Optimize the modification of glass surfaces;
- Culture both cell types (3T3 fibroblasts and PC12 cells) on the micropatterns.

1.2 Contributions of the Work

This project involved several different aspects. CMC-MA was synthesised. CMC is a semi-synthetic cellulose-derived biopolymer in which the hydroxyl groups of the glucose rings are ether-linked to carboxymethyl groups (-OCH2-COO^-). This modification renders CMC hydrophilic and water-soluble. To render CMC photoreactive, CMC was modified with aminoethyl methacrylate (AEM) to yield CMC-MA conjugates. CMC-MA was also conjugated with a fluorescent dye, 5-aminoeosin, so that the CMC-MA gels and patterns could be easily identified under a fluorescent microscope. This step was necessary so that it was possible to evaluate when the CMC-MA patterns were fully degraded, and when to culture the second cell type. Studies were made regarding the degradation of CMC-MA hydrogels under cellulase. These studies were done in two different ways: using macro CMC-MA hydrogels, and monitoring the weight loss every 1-2 h under different concentrations of cellulase; and using thin layer of CMC-MA and observing their changes over time under different concentrations of cellulase, using a

fluorescent microscope. The same experiment was conducted with PEG hydrogels, to prove that this polymer is not degraded by cellulase. Cell viability studies under cellulase were conducted. Cells were cultured in media with different concentrations of cellulase and after 48 h the culture was imaged under a fluorescent microscope to observe weather the cells survived the concentrations of cellulase used. Extensive work was done regarding the activation of glass coverslips. Glass coverslips (18 mm) were chemically modified with γ-methacryloxypropyltrimethoxysilane to allow covalent linkage of the methacrylate gels to the glass surface. Several procedures were tested to evaluate which one provided greater modification to the glass surface. The extent of glass modification was evaluated by the period of time the gels (PEG and CMC) remained attached to the glass. Finally, patterns of PEG-DM and CMC-MA were produced on glass slides. 3T3 fibroblasts were cultures on the slides, and the CMC-MA part of the pattern was degraded with cellulase, so it was possible to culture PC12 cells.

1.3 Organization of Thesis

This thesis is divided into several sections.

The first section corresponds to a general introduction, where tissue engineering is introduced, the properties of most hydrogels are discussed, and the main idea of this project is described.

The next section is the State of the Art, where the most recent advances in cell co-cultures and micropatterning are presented.

On the next section, Technical Description, the methods and procedures used in the different stages of work are described, including the procedures for the synthesis of CMC-MA and its conjugation with 5-aminoeosin, the method used to study the degradation of CMC-MC and PEG under cellulase, the studies that were conducted regarding the cell viability of Balb/3T3 fibroblasts under cellulase. The different methods that were experimented to modify the surface of glass coverslips are also presented in detail, as well as the final experiments with CMC-MA and PEG-DM patterns on glass slides.

One of the most important sections of the thesis is the next section, Results and Discussion, where the main results are presented and discussed.

The main Conclusions of the project are presented on the next section and finally a general evaluation of the work conducted can be found, including a commentary on the realization of the different objectives, as well as suggestions for future work on the subject of this thesis.

On the appendixes of the thesis, more information can be found, like pictures of CMC-MA thin layers that were not necessary on the main body of the thesis. There is also a section where a

second project that did not allow obtaining any results is presented. A more detailed description of some procedures, like culturing and staining cells, can be found in Appendix 3 and in Appendix 4 additional information is presented, regarding meetings and presentations during the semester, as well as conferences where this project will be presented.

2 State of the Art

The recent development of microfabrication tools that serve to define the cellular microenvironment has greatly facilitated the engineering of tissues for *in vitro* applications.

Conventional monolayer cultures are generated by randomly seeding cells onto homogenous substrates. Through the use of selective surface modification, microfabrication tools are now used to fabricate heterogeneous surfaces that offer control over cell-ECM and cell-cell interactions with micrometer precision (Khetani and Bhatia, 2006).

Pioneering work by Chen et al. (1997) demonstrated that the extent of spreading across a rigid substrate provided a physical cue for regulating cell fates. In addition to that, micropatterning has proven to be an enabling tool on specifying the interaction between neighbouring cells, both homotypic (same cell type) and heterotypic (between different cell types). For example, homotypic interactions between two neighbouring cells were precisely investigated using microfabricated "bowtie" wells that decouple cell-cell contact from cell spreading (Nelson and Chen, 2003). Microfabrication tools have also been utilized to evaluate the relative roles of homotypic and heterotypic interactions, thought to be important in the liver, between hepatocytes and non-parenchymal cells (Bhatia et al., 1998; Bhatia et al., 1999).

Previous studies with micropatterned co-cultures were based in the selectivity of two distinct cell types to adhere to different bioactive regions of the material.

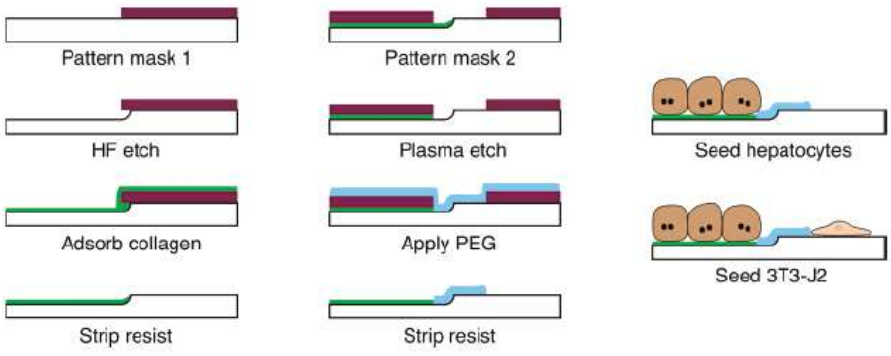

Figure 3. Schematic of patterning process where cells adhere selectively to different regions of the material (Hui and Bhatia, 2006).

The scheme on Figure 3 shows a pattern composed of two different adhesive regions that can be employed to form patterned co-cultures of two different cell types, as long as one cell

type selectively attaches to a specific region. In this work, the group started by patterning a mask in a part of the coverslip, and then the substrates were etched to remove approximately half a micrometer of the glass in the exposed area. After that, the substrates were incubated in collagen and the mask was removed. They patterned the second mask and applied plasma to remove collagen from the exposed regions. The substrates were immersed in PEG and after that, the mask was removed. They could then, culture one type of cells in the collagen surface and the second type of cells in the free glass surface. (Hui and Bhatia, 2006)

However, these micropatterns can not be used if we desire to study the interaction between two types of neurons, for example, or any two cell types that do not adhere selectively to different regions of the material.

As opposed to that, we hope that with our work, it will be possible to co-culture any two cell types, even if they do not adhere selectively to different regions of the material.

Laser micro-stereolithography (μSL) has become an accepted fast prototyping method that allows the 3D microfabrication of solid models from images created by computer aid design (CAD) programs (Maruo and Ikuta, 1998; Sachlos and Czernuszka, 2003; Zhang et al, 1999). There has recently been reported by Lu et al. (2006) a layer-by-layer laser microfabrication method for creating spatially patterned scaffolds using photo-crosslinkable polymers. This method uses a digital micro-mirror device (DMD)-based scaffold fabrication technique that allows precise, predesigned patterning of multiple molecules and allows generation of complex architectures in a layer-by-layer fashion.

This group has developed a DMD-based dynamic mask for the simultaneous photo-polymerization of partial and entire layers of a scaffold via projection. The micro-stereolithographic system was developed based on a commercial projector coupled with a digital micro-mirror device. Similar to a conventional stereolithography process, this technique creates 3D microstructures in a layer-by-layer fashion. The shapes of the constructed layers are determined by slicing the desired 3D scaffold design into a series of evenly spaced planes. Patterns of each layer are drawn in a series of PowerPoint slides, which are then executed on the DMDchip to generate a dynamic mask. The illuminated light is modulated according to the defined mask on the DMDchip and then goes through a reduction-projection lens assembly to form an image on the surface of the polymerizable resin or macromer solution. The illuminated area is solidified simultaneously under one exposure, while the dark regions remain in the liquid phase. After one layer is patterned, the substrate is lowered and the as-patterned layer is then covered by fresh macromer solution. Microstructures with complex geometries are created by sequentially polymerizing the layers. (Lu et al., 2006)

The DMD stereolithographic system is schematically shown in Figure 4. The system consists of five major components: a DMD chip embedded in the projector as a dynamic mask, a light source, a projection lens assembly, a translation stage with a micrometer, and a vat containing macromer solution. All the components cooperate to ensure correct exposure, resolution, and layer thickness.

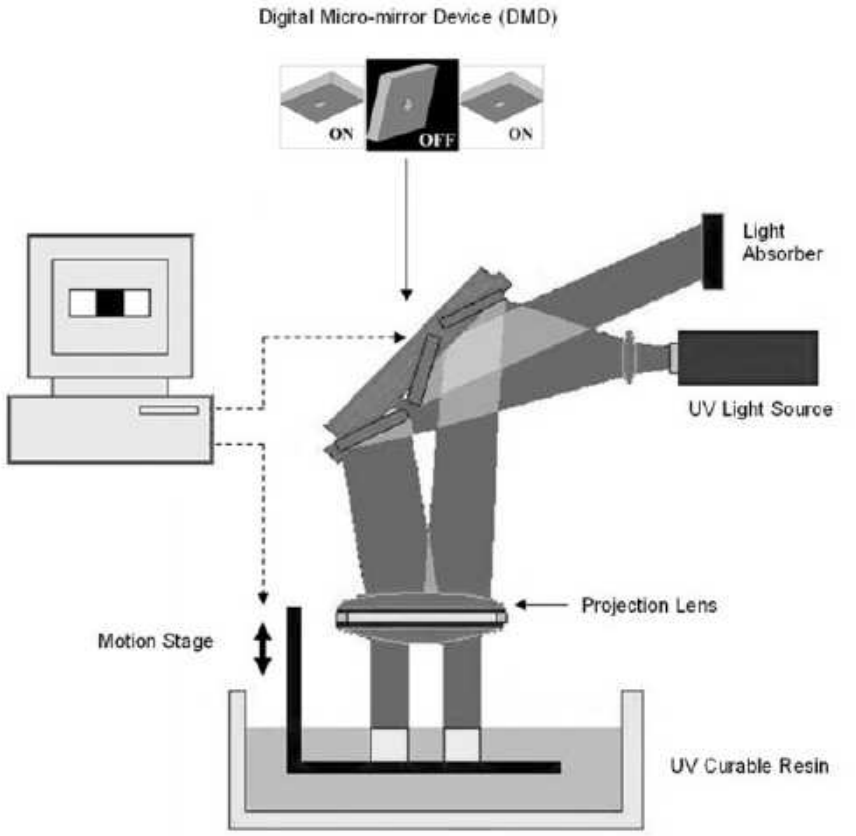

Figure 4. Schematic of the Digital Micro-mirror Device Micro-stereolithography (DMD-μSL) set up. (Lu et al., 2006)

As a biological tool, multicomponent patterning has potential application to the study of many aspects of heterotypic intercellular communication. These aspects include contact-mediating signalling, soluble signalling over various distances and functional variations within a homogeneous cell population dependent on the distance from the heterotypic contact interface. (Hui and Bhatia, 2006)

3 Technical Description

3.1 CMC-MA synthesis

This synthesis consists in the modification of CMC with aminoethyl methacrylate to yield CMC methacrylate conjugates (CMC-MA). Figure 5 depicts a schematic of the synthesis reaction. Table 1 presents other information regarding CMC.

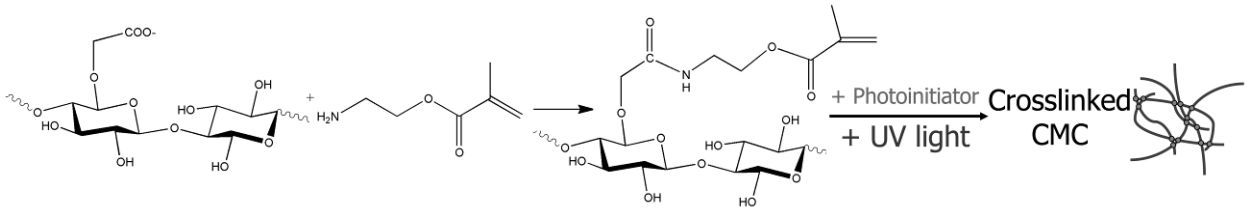

Figure 5. Modification of CMC with aminoethyl methacrylate (AEM).

AEM is a bifunctional active monomer; the amine functional group is covalently bound to CMC's carboxyl groups, while AEM's methacrylate groups are left free to participate in the photopolymerization reaction.

Table 1. Information regarding CMC.

CMC:	90 kDa
Sigma product number:	419273
Mol –COOH per mol CMC:	0.7
Average molecular weight of CMC repeat:	379

A 50 mM solution of sodium bicarbonate was prepared by adding 2.1 g of sodium bicarbonate (Sigma Aldrich) to 450 mL of deionised water. The pH was adjusted to 8.5 and more deionised water was added to make a total volume of 500 mL.

A 0.5% solution of CMC (Sigma Aldrich) in sodium bicarbonate buffer was prepared. The CMC was allowed to dissolve overnight at room temperature.

On the next day aminoethyl methacrylamide hydrochloride (AEM, Acros) was added to the CMC solution in a 2 molar excess relative to the number of CMC-COOH groups.

Because the AEM contains hydrochloride, it was necessary to adjust the pH back to 8.5, using a 1M solution of NaOH.

N-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (EDC, TCI America) was dissolved in the CMC solution, also in a 2 molar excess relative to the number of CMC-COOH groups.

In order for the synthesis reaction to occur, AEM's primary amine (pKa 8.1) must be unprotonated. Therefore, the reaction must be kept above pH 8.1. However, the EDC/CMC reactive intermediate is unstable at high pH. For these reasons, the reaction was carried out in a pH 8.5 buffer.

The reaction flask was covered with foil and left for 2 h with stirring.

After 2 h, the addition of AEM, the pH adjustment and the addition of EDC were repeated and the reaction flask was left with stirring for another 2 h.

The product was then precipitated in acetone. The volume of the reaction was noted and 5-10 times this volume of acetone was poured into a large beaker. The reaction mixture was slowly poured into the acetone with fast mixing. The product precipitated, the stirring was removed and the CMC-MA was allowed to settle. The excess acetone was decanted and the remaining acetone with CMC-MA was put into 50 mL conical tubes. These tubes were placed in the centrifuge for 10 min at 3000 rpm (rotor radius 19 cm). The excess acetone was decanted and the CMC-MA was allowed to dissolve overnight in a volume of deionised water equal to the initial volume of the reaction noted.

In the next day, the product was dialysed against deionised water in the dark at room temperature for approximately 24 h. The deionised water dialysate was changed 4-5 times (more frequently at the beginning of the dialysis).

The product was then lyophilized for 3 days and stored in the dark at -20 ºC.

3.2 CMC-MA conjugation with 5-amimoeosin

CMC-MA may be conjugated with fluorescent dyes, such as 5-aminoeosin (Invitrogen), so that the CMC-MA gels and patterns may be easily identified under a fluorescent microscope. Since 5-aminoeosin has a primary amine, the reaction is similar to the addition of the aminoethyl methacrylate to CMC. Sulfo-NHS (Pierce) was used to increase the efficiency of the reaction. Figure 6 presents the conjugation scheme of CMC-MA with 5-aminoeosin.

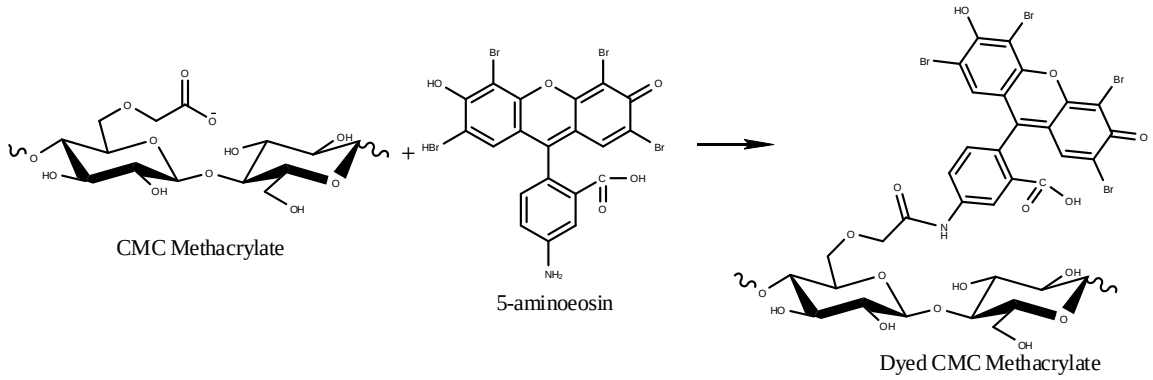

Figure 6. Modification of CMC-MA with 5-aminoeosin.

A 0.5% solution of CMC-MA in 50 mM sodium bicarbonate buffer was prepared, by adding 0.4 g of CMC-MA, very slowly and under rapid mixing, to 80 mL of sodium bicarbonate buffer. The CMC-MA was allowed to dissolve overnight.

In the next day the 5-aminoeosin was added to the CMC-MA solution in a 0.1 molar ratio relative to the number of CMC-COOH groups. The EDC was then added to the solution in a 2 molar excess relative to the number of CMC-COOH groups. Finally, Sulfo-NHS was dissolved, such that its molarity was 5 mM. The reaction flask was covered with foil and left with stirring for 4 h at room temperature.

The product was then precipitated in acetone as described for the CMC-MA synthesis.

The product was dialysed against deionised water in the dark and at room temperature for 24 h and then lyophilized for 3 days.

Finally, the dyed CMC-MA was stored in the dark at -20 ºC.

3.3 Degradation of CMC-MA under cellulase

3.3.1 Degradation of macro CMC-MA hydrogels under cellulase

The steps that relate to the micropatterns are: (1) culture 3T3 fibroblasts; (2) degrade the CMC-MA patterns to provide new adhesive sites for the cells to adhere; (3) culture PC12 cells. It was therefore necessary to study the degradation of CMC-MA under different concentrations of cellulase, to be able to determine the optimal concentration of enzyme to be used in the cell culture experiments.

At first these studies were conducted on macro CMC-MA hydrogels (80 μL). The first step was to prepare the hydrogels. A 0.05% solution of Irgacure 2959 (Ciba Specialty Chemicals) in phosphate buffer saline (PBS) was prepared. This solution will be stable for several weeks if

stored in the refrigerator in the dark. Three solutions (4, 8 and 12%) of CMC-MA in 0.05% Irgacure 2959 were prepared. The CMC-MA is not easily dissolved in the Irgacure solution, so a vortex mixer was used to promote the dissolution. After 10-30 min in the vortex mixer, the CMC-MA was completely dissolved but the solution had air bubbles. To remove those bubbles, the solution was placed in a centrifuge, at 380 rpm for 2 min. The CMC-MA solutions are very viscous and hard to work with; a micropipette was used to measure and dispense the solutions. 80 µL of CMC-MA solutions were placed in a mold to form each of the hydrogels. The solutions were placed under UV light (wavelength 365 nm) for approximately 2 min. Nine hydrogels of each CMC-MA concentration were prepared.

The hydrogels were stored in PBS in 12-well plates overnight to swell and desorb excess reactants. In the next morning, the PBS was removed from the 12-well plates with a syringe and from the hydrogels with absorbent paper. The hydrogels were weighed and returned to the 12-well plates. The plates were filled with solutions of cellulase (Sigma Aldrich) in cell culture media, with different concentrations (0.2, 0.5 and 1.0 U/mL). The work was done with the 12-well plates configured as shown in Figure 7. Next, the weight loss of the hydrogels was measured every 1-2 h for the next 48 h, except during the night. Every time the weight loss was measured, the plates were replenished with fresh cellulase solution and incubated at 37 ºC. Degradation rates were calculated from the initial linear slope of gel weight loss versus degradation time plots.

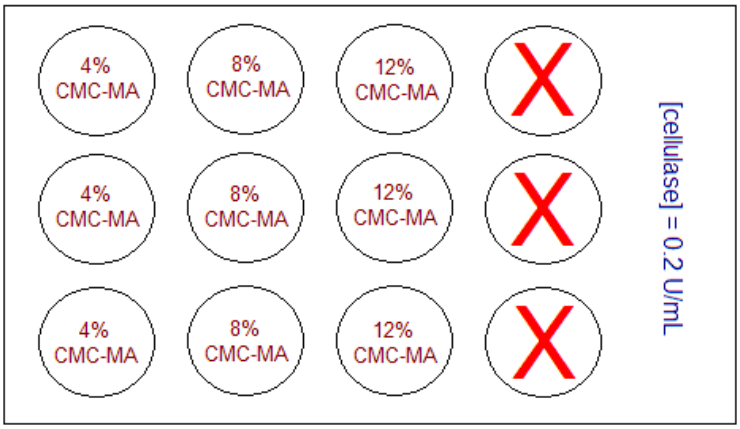

Figure 7. Configuration of the 12-well plate for 0.2 U/mL cellulase during the degradation studies (two other plates were used for 0.5 and 1.0 U/mL, with the same configuration).

This experiment was repeated twice, however, only 8 and 12% CMC-MA hydrogels were used on the second and third experiments, and the masses of the gels were only monitored during the first day (≈9 h).

3.3.2 Degradation of CMC-MA thin layers under cellulase

Since the micropatterns are much smaller than the macro hydrogels that have been used for the degradation studies, it was decided to also study the degradation of thin layers of CMC-MA under cellulase, because the behaviour of these hydrogels is expected to approximate that of the micropatterns.

The thin-layer degradation studies were also conducted with 8 and 12% CMC-MA exposed to 0.2, 0.5 and 1.0 U/mL cellulase. The procedure was the same as the previous degradation studies, except that the solutions of CMC-MA were crosslinked on top of activated glass coverslips, so that they would remain attached to a stable substrate. On top of the solution, another glass coverslip was placed to spread the solution and form a thin layer (≈0.5 mm thick and 12 mm of diameter). A schematic of this procedure can be seen in Figure 8.

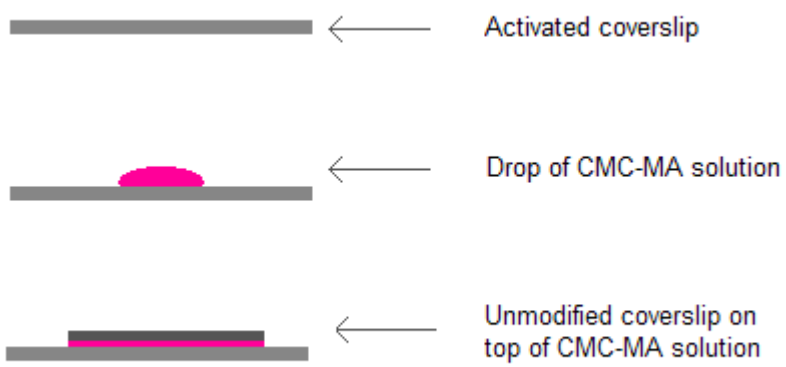

Figure 8. Schematic of the preparation of thin layers of CMC-MA.

During these experiments, the masses of the thin layers of CMC-MA was not monitored, because these hydrogels are very thin and weigh much less than the coverslip they are attached to, so changes in mass would be difficult to measure with precision. For this reason, the results of this experiment are qualitative. These qualitative results were obtained by imaging the thin layers under an inverted fluorescent microscope. In the first of these experiments, it was intended to image the layers after 24 h under cellulase. However, after those 24 h, some of the layers had already been completely degraded. Because of that, it was decided to repeat the experiment but this time the layers were imaged every 3 h, and after 24 h.

Some scratches were made on some of the thin layers during the first of these experiments, because the micropatterns of CMC-MA to be degraded are not continuous, and this way it is expected to verify if this has any influence on the degradation of the CMC-MA patterns

3.4 Degradation of PEG under cellulase

The procedure for this experiment is similar to the degradation studies of CMC-MA under cellulase.

Macro PEG-DA (poly(ethylene glycol) – diacrylate) and PEG-DM hydrogels were prepared. 20% solutions of PEG-DA and PEG-DM in 0.05% Irgacure 2959 were prepared. 80 µL of these solutions were placed on a mold to form each of the hydrogels. The solutions were placed under UV light (wavelength 365 nm) for approximately 8 min. Nine hydrogels of each kind of PEG were prepared.

The hydrogels were stored in PBS in 12-well plates overnight to swell and desorb excess reactants. In the next morning, the PBS was removed from the 12-well plates with a syringe. The hydrogels were weighed and returned to the 12-well plates. The plates were filled with solutions of cellulase (Sigma Aldrich) of different concentrations (0.2, 0.5 and 1.0 U/mL). The work was done with the 12-well plates configured as shown in Figure 9. After that, the weight loss of the hydrogels was measured every 1-2 h for the next 10 h. Every time the weight loss was measured, the plates were replenished with fresh cellulase solution and incubated at 37 ºC.

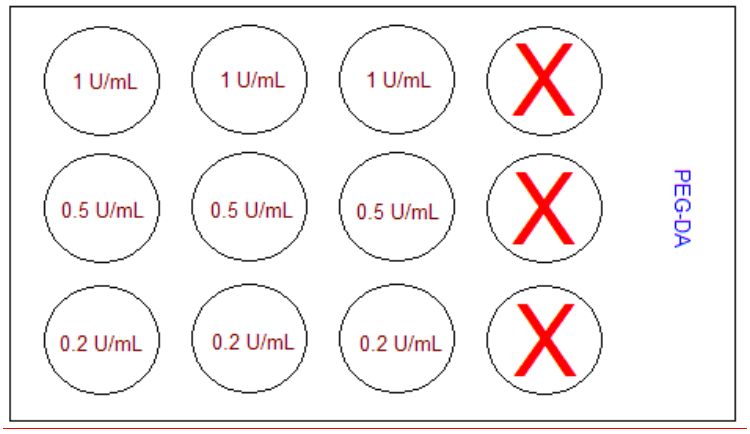

Figure 9. Configuration of the 12-well plate for PEG-DA during the degradation studies of PEG (another plate was used for PEG-DM with the same configuration).

3.5 Cell viability under cellulase

The viability of Balb/3T3 fibroblasts (ATCC) was tested in medium containing 0.2-1.0 U/ml cellulase and analyzed from phase microscopy (Olympus) images captured at 48 h. Glass activated coverslips (12-mm diameter) were placed in 24-well plates and 500 µl of cell culture medium containing cells at a cell density of 50 000 cells/ml were added to each well. The cells were allowed to attach and proliferate for 24 h in the absence of cellulase in the medium. After 24 h, cellulase was added to the cell culture medium at the same concentrations used for the degradation experiments and the cells were incubated for another 48 h. Every 1-2 h (except during the night) the cells were replenished with fresh pre-warmed medium containing cellulase and the cells were visualized after 48 h of culture.

This experiment was repeated, but the second time, a live/dead assay was conducted, so that it could be possible to quantify the percentage of dead cells in each concentration of cellulase. 1 mL of cell culture medium containing cells at a cell density of 50 000 cells/ml was added to each well on a 12-well plate. 3,3'-dioctadecyloxacarbocyanine ($DiOC_{18,}$ Invitrogen) was added to the cell culture medium. This is a green fluorescent membrane stain that will label all cells. The cells were allowed to attach and proliferate overnight in the absence of cellulase in the medium. After that, cellulase was added to the cell culture medium at the same concentrations used for the degradation experiments and the cells were incubated for another 24 h. Every 1-2 h (except during the night) the cells were replenished with fresh pre-warmed cellulase containing medium. After 24 h under cellulase, propidium iodide (PI, Invitrogen) was added to the cell culture medium and the 12-well plate was incubated for another 15 min. This dye is membrane-impermeant and will label any dead cells due to their compromised plasma membranes. The cells were visualized after 24 h of culture in medium with cellulase.

3.6 Modification of glass coverslips

This step of the project presented the most challenges because new procedures were required to be developed and optimized. For that reason, several procedures to modify glass surfaces with methacrylate groups were explored. These procedures are listed and described in the next paragraphs.

For glass surface modification, usually vinyl silane or methacrylate silane are used as coupling agents, because they contain at least one functional group with an alkene, which can readily react with another alkene-containing polymer, as well as functional groups that react with silanol groups on the glass surface (Vidic et al., 2005). In all the procedures described below,

the silane used was γ-methacryloxypropyltrimethoxysilane (or trimethoxypropylsilanemethacrylate, Sigma Aldrich).

Method 1A

The first method used for the modification of the glass coverslips was carried out according to the procedure given by the manufacturer's product information:

Glass coverslips were cleaned with soap and rinsed thoroughly with water and dried. 100 mL of a 0.5% solution of γ-methacryloxypropyltrimethoxysilane in ethanol was prepared and 3 mL of dilute acetic acid were added (1:10 glacial acetic acid:water). The glass coverslips were placed in this solution and let rest for about 3 min. The last step was to pour off the excess solution, and rinse the coverslips with ethanol to remove the residual reagent. The glass coverslips were allowed to dry thoroughly at room temperature.

Method 2A

A similar method was found in the literature (Tsang et al., 2007) and provided the basis for optimization of Method 1A:

As described in literature (Tsang et al., 2007), a 2% solution of silane in 95% ethanol was used instead of the previous 0.5% solution in ethanol. Also, instead of adding 3 mL of acetic acid, the pH of the solution was adjusted to 5, by adding acetic acid. Finally, instead of letting the glass coverslips dry at room temperature, they were placed in an oven at 110 ºC for 5-10 min.

Methods 1B and 2B

The method found in literature (Tsang et al., 2007) also used an ethanol wash to clean the coverslips rather than soap. In Methods 1B and 2B, this revised washing step was incorporated into the Methods 1A and 2A described above.

Method 3

Chromic sulphuric acid (Fisher Scientific), a common agent used to prepare substrates for microelectronics pattern technologies was also investigated for the initial cleaning step:

The glass coverslips were rinsed individually with ethanol and then they were placed in a beaker with ethanol. The beaker was put in an ultrasonic bath for 15 min. The ethanol was drained and the coverslips were washed with deionised water. The beaker was filled with

water and placed again in the ultrasonic bath for 10 min. The water was removed and the coverslips were put in the oven at 110 ºC for 2 h. When the coverslips were dry, they were placed on chromic sulphuric acid overnight. In the next day the acid was removed and the coverslips were washed with deionised water. They were then placed in water and in the ultrasonic bath again for 10 min. Finally, the water was removed and the coverslips were put in the oven at 110 ºC to dry. After that, the coverslips were activated using the previous methods.

Method 4

Some new research was made in the literature regarding the activation of glass coverslips using γ-methacryloxypropyltrimethoxysilane, and a new method was found, which was thought that might improve the attachment of the micropatterns to the glass (Vidic et al. 2005).

In this work, Vidic et al. (2005) explored various glass pre-treatments, using different methods of surface activation with silane and a number of drying methods. They also explored a range of glass types. All together they performed over 80 experiments, combining the different methods for these three steps. To evaluate the efficiency of the glass surface treatment procedures, the quantity of silane groups was determined by measuring the contact angle between the glass surface and a water droplet. This method is based on an increasing of hydrophobicity after silane binding as a result of hydrophobic double bonds present on vinyl silane. The higher the contact angle, the higher the surface hydrophobicity, which is associated with a higher amount of double bonds present, and consequently a higher strength of attachment is expected. Therefore, the quality of surface treatment was checked by the difference in contact angle of water on silanized and unsilanized glass surface.

One of the three glass types tested in this study is the glass that constitutes the coverslips that were used on all the experiments for this project, borosilicate glass. So, it was decided to try the procedure found in the article which allowed the authors to obtain the greater angle difference for that type of glass:

Glass coverslips were washed individually with ethanol and deionised water. They were placed in a beaker with a 0.1 M solution of NaOH for 30 min in an ultrasonic bath. After that, the coverslips were rinsed with ethanol and placed to dry overnight at 110 ºC. On the next day, the glass coverslips were immersed in a 30% solution of silane in acetone for 24 h at 25 ºC. They were then rinsed with acetone and placed to dry overnight in a vacuum desiccator at room temperature.

3.7 Micropatterns

Our collaborator from the University of Oklahoma, School of Industrial Engineering, Dr. Binil Starly, sent two sets of micropatterns, which were imaged using an inverted microscope (Olympus) using phase optics.

A micro-stereolitographic system (μSL) based on a digital micro-mirror device (DMD™, Texas Instruments) was used to create the patterns. A PEG-DA solution was formulated by dissolving PEG-DA (700 kDa) in water at a final concentration of 60 - 80% (w/v). 300 μL of photoinitiator solution (20% of 2,2-Dimethoxy-2-phenyl-acetophenone in 1-Vinyl-2-pyrrolidinone) were added to 10 mL of polymer solution. The polymer was crosslinked under UV light on top of the activated coverslips and the patterns were obtained in a layer-by-layer fashion by modulating the illuminated light according to a defined mask on the DMD chip (Lu et al., 2006).

On the first set of micropatterns received, Balb/3T3 fibroblasts were cultured at a cell density of 50 000 cells/mL for 24 h. This allowed assessing cell compatibility of the micropatterns.

3.8 CMC-MA/PEG-DM patterns

Macro CMC/PEG patterns were produced on top of glass slides. The slides surface was modified using Method 2B.

The first procedure that was tried to produce the macro CMC/PEG patterns involved using a polydimethylsiloxane (PDMS) mold, attaching this mold to a square piece of glass using a plasma cleaner, coating the PDMS mold with CMC-MA using a spin coater, pressing the mold (CMC pattern down) against the activated glass slide, and placing under the UV light to crosslink the CMC-MA. The PDMS mold would then be removed and the CMC-MA patterns would remain attached to the activated glass. The procedure is repeated with PEG-DM, yielding a CMC/PEG pattern.

However, on the first attempt to follow this procedure, it was possible to verify that the plasma cleaner was not working, so it was necessary to come up with an alternative.

After that, a procedure similar to the one used to produce the thin layer of CMC-MA for degradation studies was tried.

Figure 10 depicts an overview of the CMC/PEG patterning procedure. A drop of PEG-DM (15 μL) was placed near one of the corners of an activated glass slide. A glass coverslip was placed on top of the drop, and slight pressure was applied to form a thin layer between the glass slide and the glass coverslip. The slide was placed for 4-5 min under the UV light to

crosslink the PEG-DM. Next to that corner, the procedure was repeated using a CMC-MA solution of 8% (40 µL drop). The slide was placed again under UV light to crosslink the CMC-MA and the PEG-DM.

This procedure was done on both sides of the glass slide.

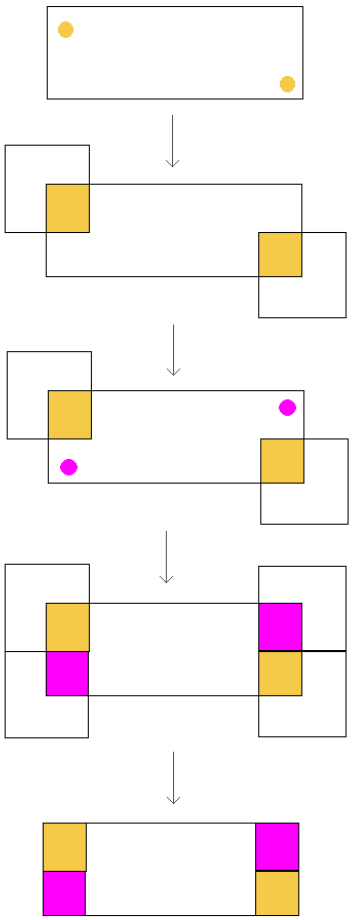

Figure 10. Schematic of the procedure for the production of the macro CMC/PEG patterns. Orange and pink denote PEG-DM and CMC-DA, respectively.

After the patterns on the glass slides were prepared, the samples were placed in 10 cm tissue culture plates and Balb/3T3 fibroblasts were cultured on the samples at a cell density of 50 000 cells/mL. The cells were allowed to adhere overnight; the next morning, the cell culture medium on the plates was replaced by cellulase containing medium, at a cellulase concentration of 0.5 U/mL, to degrade the CMC-MA pattern. Every 1-2 h the medium was replaced by fresh cellulase containing medium for the next 12 h. By the end of that time, the CMC-MA patterns were completely degraded and PC12 cells, a neuron-like cell line, were cultured at the same cell density. These cells were allowed to adhere overnight.

In the next morning, the cells were fixed on the slides using a 4% solution of formaldehyde and imaged on an inverted microscope.

This experiment was repeated another two times. The second and third times, the Balb/3T3 fibroblasts were labelled with $DiOC_{18}$ (green) before being cultured on the slides, and at the end of the experiment all the cells were labelled with DAPI (4',6-diamidino-2-phenylindole, blue).

Each time this experiment was conducted, two patterned slides were used.

4 Results and Discussion

4.1 Degradation of CMC-MA under cellulase

4.1.1 Degradation of macro CMC-MA hydrogels under cellulase

This experiment was conducted using 3 hydrogels of each CMC-MA concentration for each cellulase concentration.

To determine the degradation rates of the macro CMC-MA hydrogels under different concentrations of cellulase, the masses of the hydrogels were monitored for 48 h. The degradation rates were determined using the initial linear slope of gel weight loss versus degradation time plots. An example of these plots is given in Figure 11.

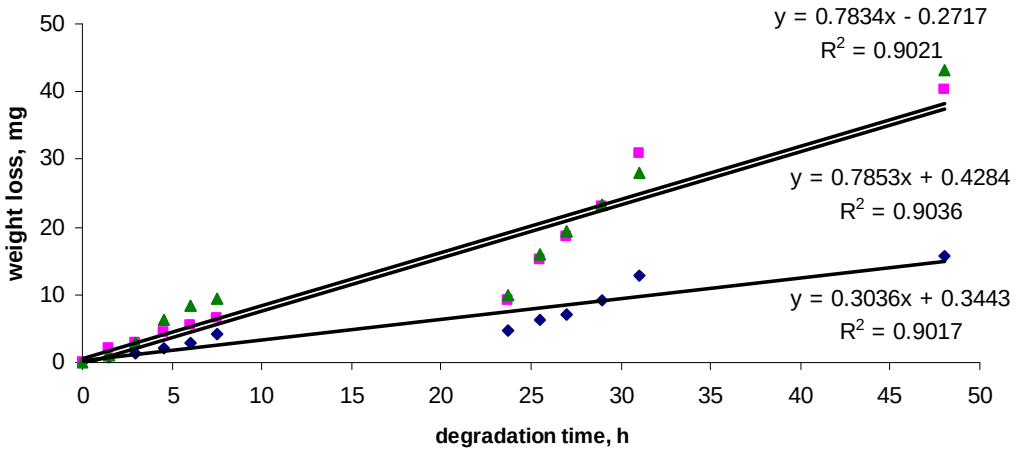

Figure 11. Weight loss of 12% hydrogels in 1 U/mL cellulase.

From these plots it is possible to determine the degradation rates of the hydrogels (weight loss (mg)/h) under different concentrations of cellulase.

Table 2. Degradation rates for different hydrogels under different concentrations of cellulase.

	4% gels			8% gels			12% gels		
	Cellulase concentration (u/mL)			Cellulase concentration (u/mL)			Cellulase concentration (u/mL)		
	1	**0.5**	**0.2**	**1**	**0.5**	**0.2**	**1**	**0.5**	**0.2**
degradation rate (mg/h)	10.95	11.94	9.64	1.26	1.58	0.99	0.78	0.56	0.31
	5.91	9.43	11.25	1.09	1.41	0.92	0.79	0.28	0.39
	6.66	3.97	6.81	1.23	1.58	0.79	0.3	0.33	0.23
Average	7.84	8.45	9.23	1.19	1.52	0.9	0.62	0.39	0.31
Std	2.22	3.33	1.83	0.07	0.08	0.08	0.23	0.12	0.06

These results were compiled in the graphic shown in Figure 12.

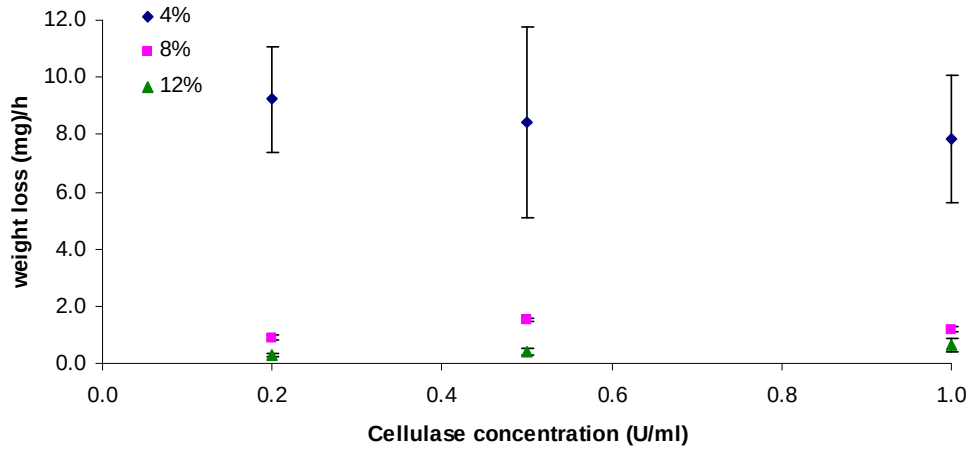

Figure 12. Degradation rates for different hydrogels (4, 8 and 12% CMC-MA) under different concentrations of cellulase.

The data for the 4% hydrogels has a large amount of error because these hydrogels are very fragile and break during the course of the experiment. This corresponds to greater mass losses due to the procedures rather than the degradation itself. Therefore, the data for the 4% hydrogels was disregarded. If only the data regarding the 8 and 12% hydrogels is considered, the graphic shown in Figure 13 is obtained.

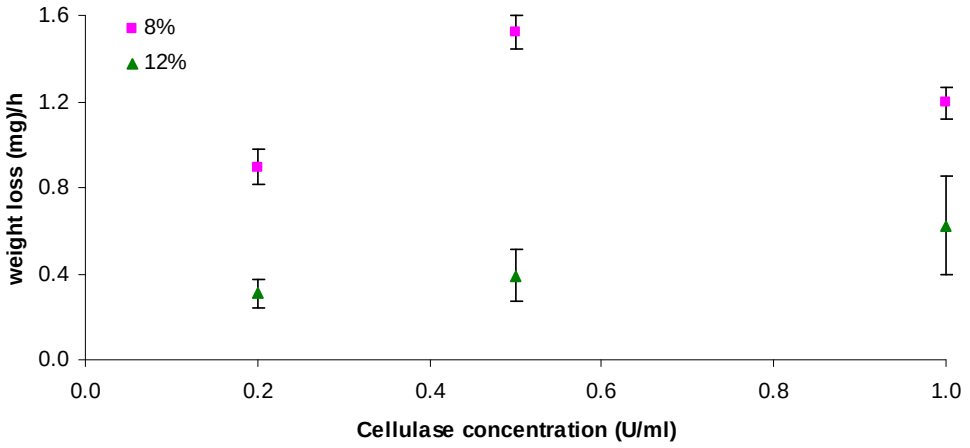

Figure 13. Degradation rates for 8 and 12% hydrogels under different concentrations of cellulase.

As expected, 0.2 U/ml cellulase degrades the gels at the lowest rate (Figure 13). Also, for the 12% hydrogels, there is not a statistically significant difference between 0.5 or 1.0 U/mL of cellulase ($p=0.13$). For the 8% hydrogels there is a statistically significant difference between the degradation rates resulting from 0.5 and 1.0 U/mL cellulase ($p=0.0006$), however, the implication of these results is unclear because the degradation rate with 1.0 U/mL is lower than with 0.5 U/mL. It was concluded that acceptable degradation rates can be obtained using either concentration. The 8% hydrogels were almost totally degraded (>90%) and more than 50% of the 12% hydrogels were degraded.

Figure 11 also indicates that the initial slope (data from the first day) is very different from the slope of the data regarding the second day (between 24 and 31 h). This occurred because in the second day the 8 and 12% gels start to break (4% gels are totally degraded by the end of the first day), and the mass losses during the procedure increase. Taking this into consideration, the degradation rates from the slope of the data of the first day alone were calculated. An example of the degradation data is represented in Figure 14.

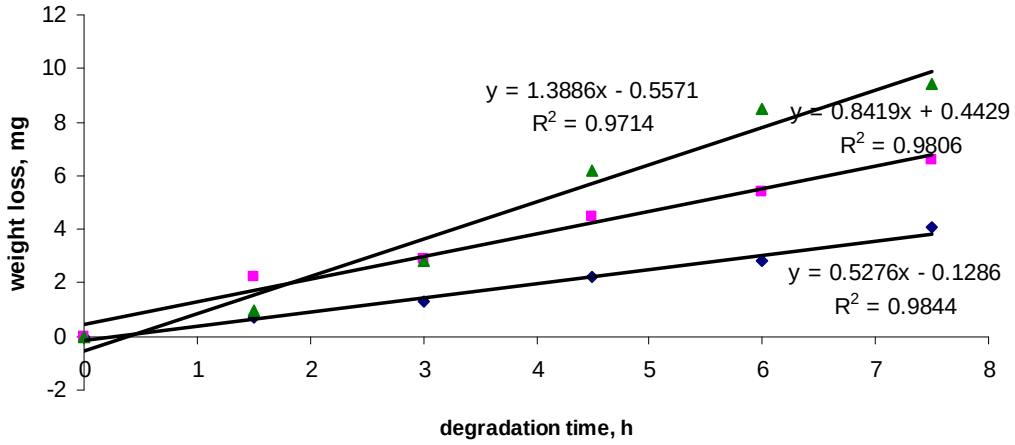

Figure 14. Weight loss of 12% hydrogels under 1 U/mL cellulase during the first day.

Using these new linear slopes it was possible to obtain a graph similar to Figure 12, but this time, using only data from the first day (Figure 15).

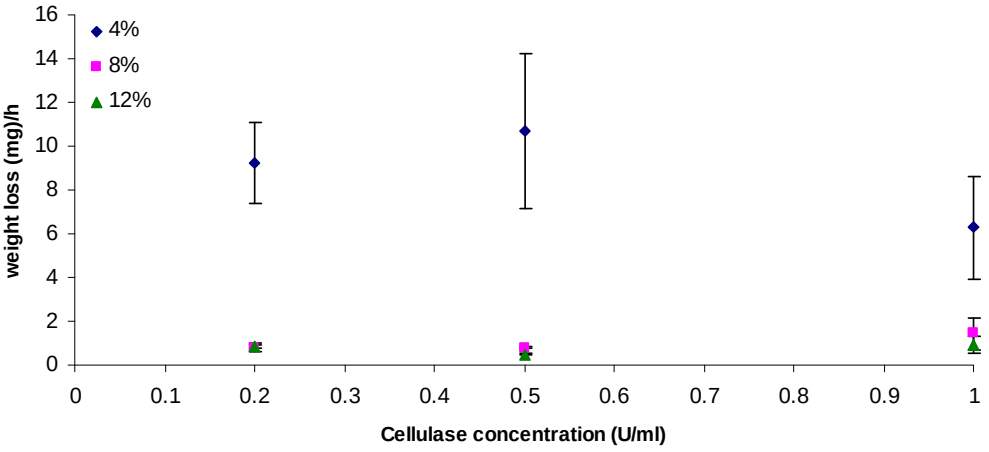

Figure 15. Degradation rates for different hydrogels (4, 8 and 12% CMC-MA) under different concentrations of cellulose (considering only data from the first day).

Once again, the results regarding 4% hydrogels are not very accurate, so only the 8 and 12% gels were considered (Figure 16).

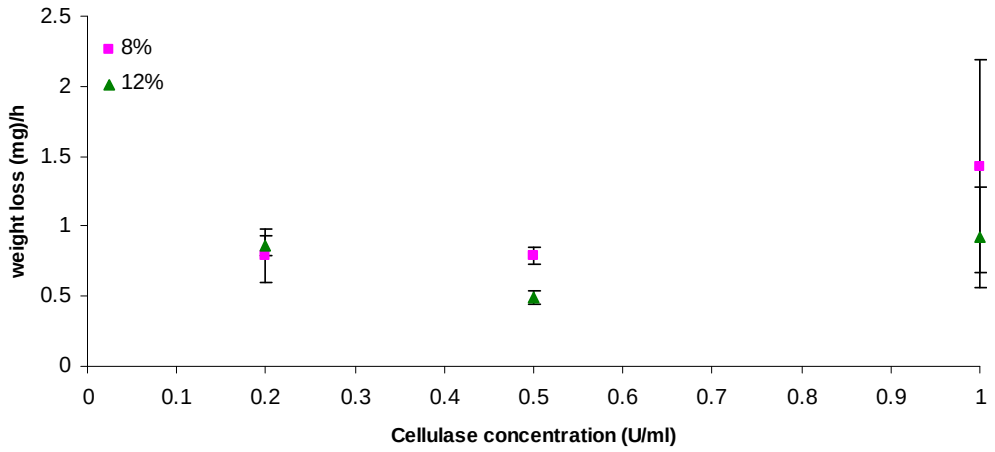

Figure 16. Degradation rates for 8 and 12% hydrogels under different concentrations of cellulase (considering only data from the first day).

The hydrogels degrade under all enzyme concentrations tested and it appears that the rate is not a function of enzyme concentration for the range tested.

This experiment was repeated another two times. However, for the second and third times, only 8 and 12% hydrogels were used, and the masses of the gels were only monitored during the first day (≈9 h).

The degradation rates obtained for the second and third experiments were plotted against the concentration of cellulase and are presented in Figures 17 and 18.

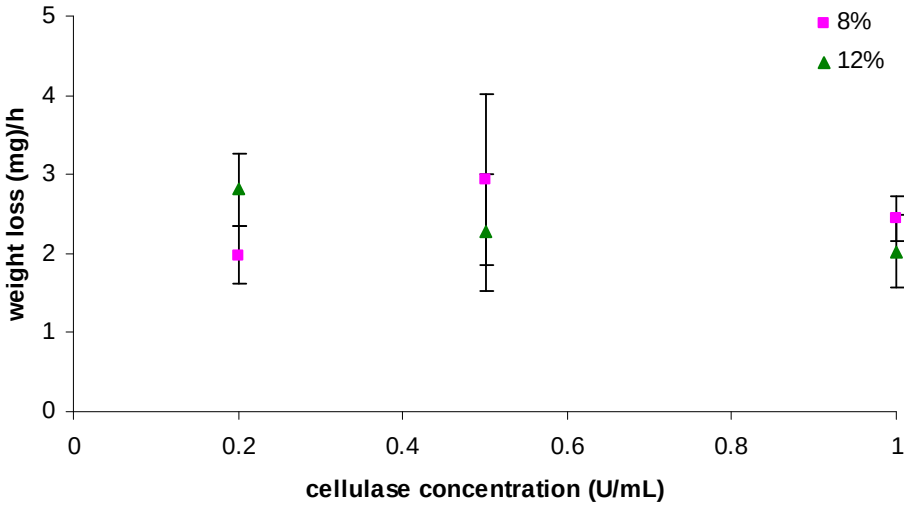

Figure 17. Degradation rates for 8 and 12% CMC-MA hydrogels under different concentrations of cellulase (second degradation study experiment).

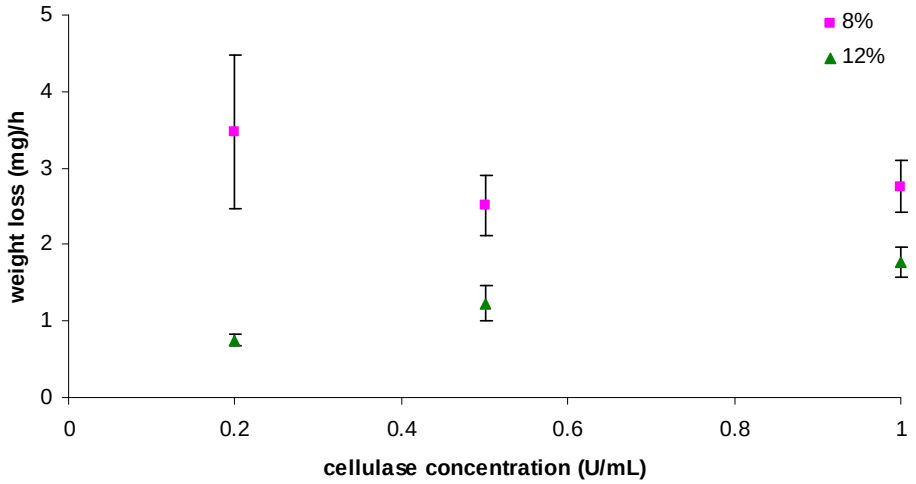

Figure 18. Degradation rates for 8 and 12% CMC-MA hydrogels under different concentrations of cellulase (third degradation study experiment).

The repeated trials confirm that the hydrogels degrade under all enzyme concentrations tested and it appears that the rate is not a function of enzyme concentration for the range tested. However, in some cases, like the 12% gels in Figures 11 and 16, the degradation rate seems to increase with the increase in cellulase concentration, which was expected.

4.1.2 Degradation of CMC-MA thin layers under cellulase

Since the thin gels are bound to coverslips and have a small mass relative to the glass, mass losses of these gels would be difficult to measure with precision, so it was necessary to rely on visual observations for this experiment.

During this experiment it was possible to visualize the degradation of the gels, especially on the border, which started to become irregular after a couple of hours.

The gels detached from the activated glass coverslips after 24 h immersed in cellulase, possibly due to the degradation.

The 8% gels degraded faster than the 12% gels, which was expected. However, after 48 h, the gels remained mostly intact, which could pose a problem for the micropatterning application (trial 1).

This experiment was repeated (trial 2), but this time dyed CMC-MA was used, to make it possible to image the gels under an inverted fluorescent microscope. Also, during this second experiment, scratches were made on some of the gels, because the micropatterns of CMC-MA are not continuous, and this simple approximation would allow to determine whether surface structure has any influence on the degradation of the CMC-MA patterns.

During the second experiment, it was intended to image the hydrogels after 24 h of cellulase activity.

During this experiment some of the gels detached from the coverslips after being left overnight immersed in PBS. This indicates problems regarding the activation of the glass coverslips. The rest of the gels detached from the coverslips after only 1-2 h under cellulase.

The 8% gels degraded faster than the 12% gels, which was expected. After 24 h the 8% gels in 1 U/mL cellulase had completely degraded. The 8% gels which were under 0.5 U/mL of cellulase had not completely disappeared, but had broken into pieces and the volume had decreased significantly. The 8% gels under 0.2 U/mL of cellulase were still whole after 24 h but the edges had started to degrade. As for the 12% gels, it was not possible to observe any significant differences after 24 h.

Regarding the hydrogels in which there had been made scratches to simulate the micropatterns, after overnight immersion in PBS, the pieces of CMC-MA had detached from the coverslips and were floating in the solution. For this reason, these gels were not included in the degradation experiments, for it would be difficult to replace the solution without large mass losses of hydrogel, making the results inaccurate.

Since some of the gels had completely disappeared after 24 h, they were not imaged under an inverted fluorescent microscope. Instead, it was decided to repeat the experiment (trial 3) and image the gels every 3 h and after 24 h.

The gels were first imaged before they were placed under cellulase. These images are presented in Figures 19 and 20 (the images on the right have a blend background).

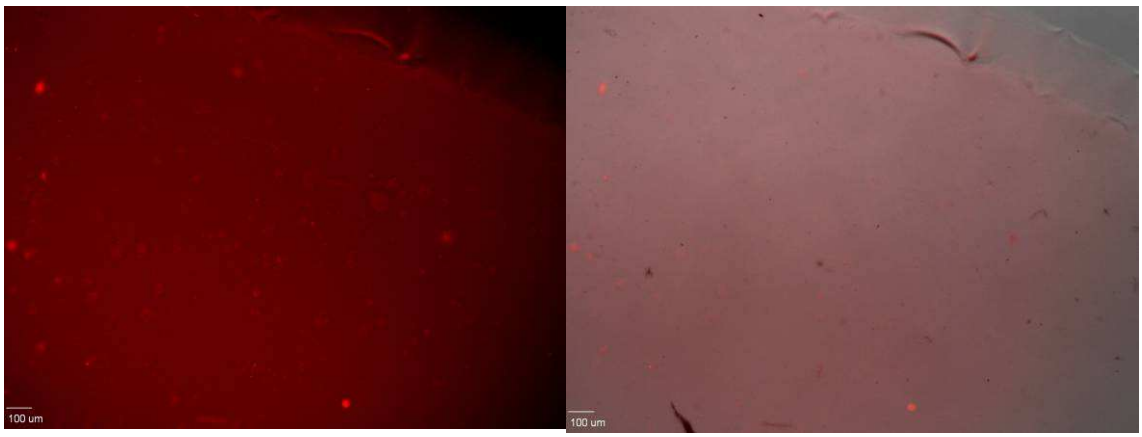

Figure 19: 8% CMC-MA hydrogels before being placed under cellulase. (scale bar – 100 µm)

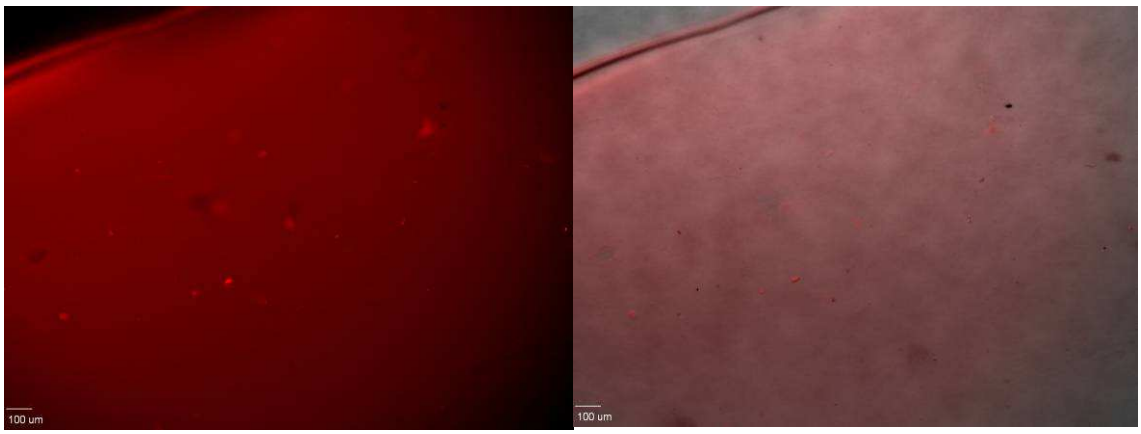

Figure 20: 12% CMC-MA hydrogels before being placed under cellulase. (scale bar – 100 µm)

After 3 h and 6 h under cellulase, the gels were imaged again. Examples of these images are presented in Figures 21 to 24.

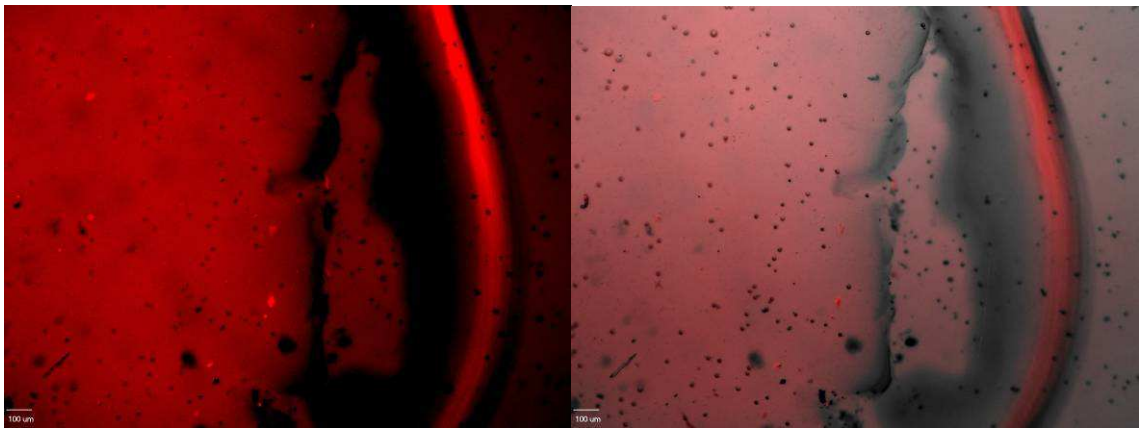

Figure 21. 8% CMC-MA hydrogels after 3 h under 1 U/mL cellulase. (scale bar – 100 µm)

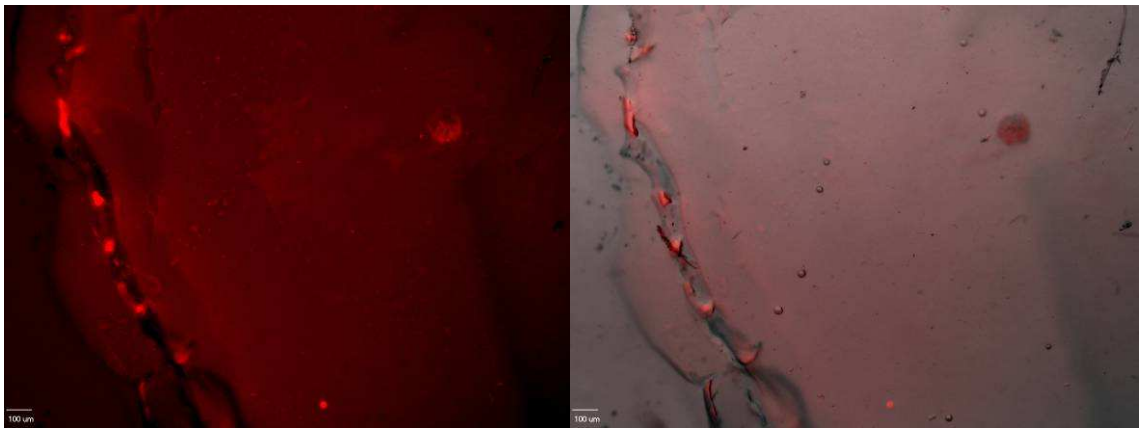

Figure 22. 12% CMC-MA hydrogels after 3 h under 1 U/mL cellulase. (scale bar – 100 µm)

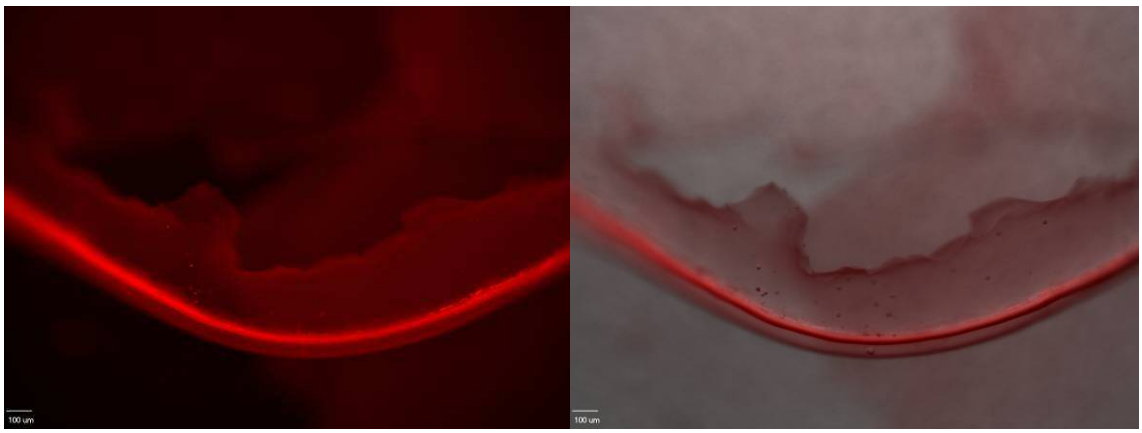

Figure 23. 8% CMC-MA hydrogels after 6 h under 1 U/mL cellulase. (scale bar – 100 μm)

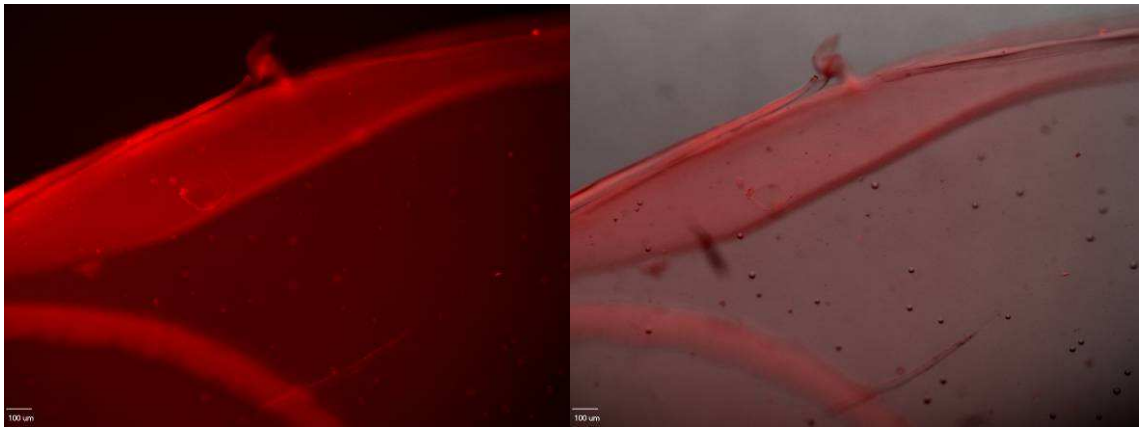

Figure 24. 12% CMC-MA hydrogels after 6 h under 1 U/mL cellulase. (scale bar – 100 μm)

Note that the gels degrade during the course of the experiment. The 8% gels degrade faster than the 12% gels, as expected, and although they seem degrade faster at 1 U/mL of cellulase, at 0.5 U/mL the degradation is also fairly fast (the rest of the images can be seen in Appendix 1).

Next, the gels were imaged again after 24 h under cellulase (Figures 25 and 26).

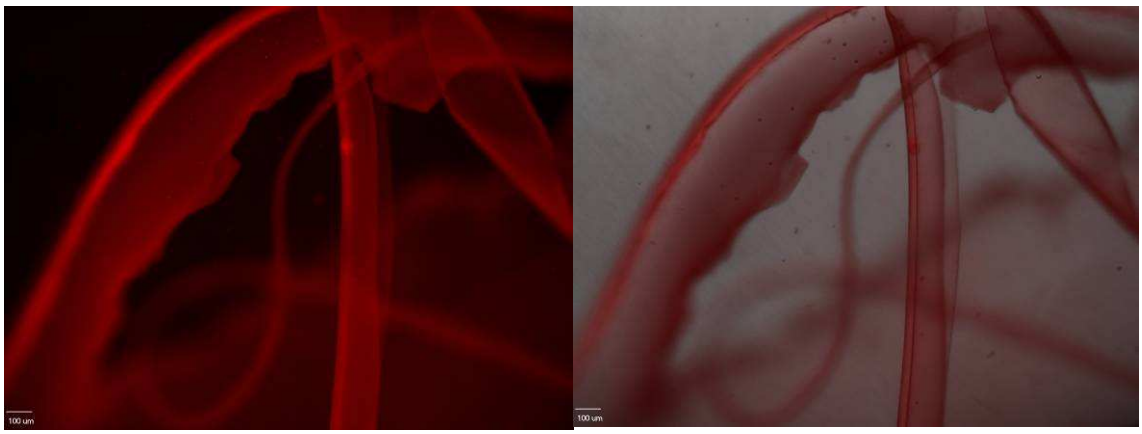

Figure 25. 8% CMC-MA hydrogels after 24 h under 1 U/mL cellulase. (scale bar – 100 μm)

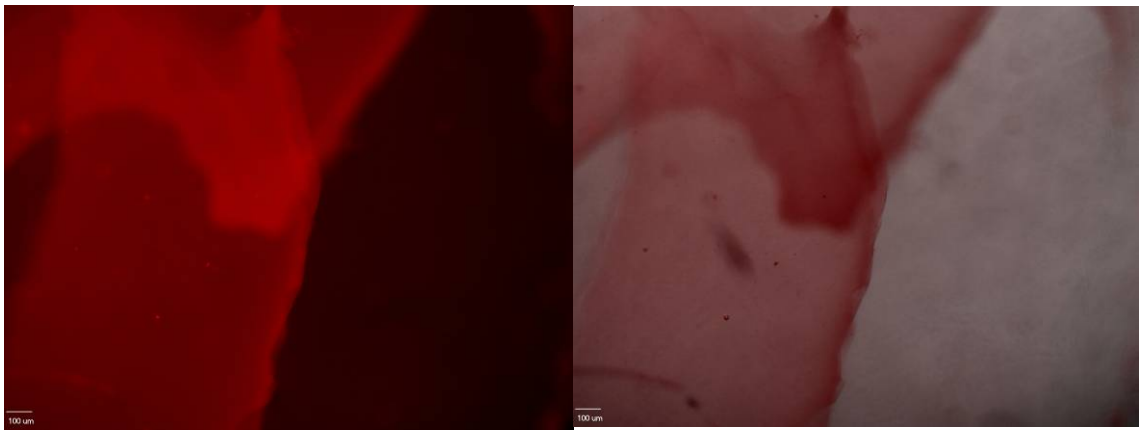

Figure 26. 12% CMC-MA hydrogels after 24 h under 1 U/mL cellulase. (scale bar – 100 μm)

In this experiment, as opposed to trial 2, the 8% gels under 1 U/mL of cellulase did not completely degrade after 24 h in enzyme solution. This may be due to the fact that during this experiment it was not possible to replace the solution of enzyme as many times as in the previous experiment. However, these gels, as well as the 8% gels under 0.5 U/mL of cellulase, are broken and the effects of the degradation are obvious in the images. These two concentrations of cellulase seem to lead to a faster degradation of the gels, than the concentration of 0.2 U/mL.

The 12% gels are still whole and the effects of the degradation are much less obvious, although also present (noticeable at the edges of the layers).

From these experiments, it was decided that for the production of the micropatterns it will be better to use 8% CMC-MA, to minimize the time of exposure of the first type of cells to the cellulase solution.

4.2 Degradation of PEG under cellulase

The degradation studies of PEG under cellulase, were carried out using three hydrogels of each type of PEG (PEG-DM and PEG-DA) in each concentration of cellulase. Figure 27 depicts the data for the three hydrogels of PEG-DM under a concentration of 1 U/mL of cellulase (hydrogel mass versus degradation time).

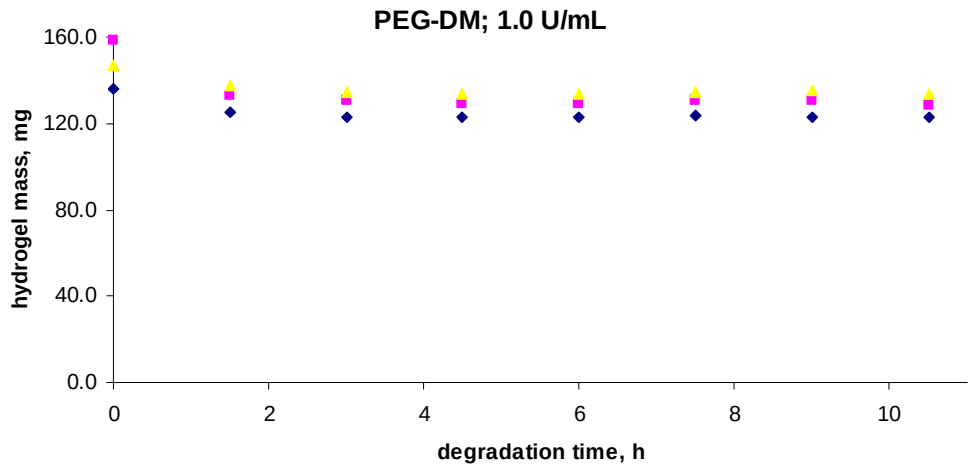

Figure 27. Masses of PEG-DM hydrogels under 1 U/mL cellulase.

This plot is presented as an example. All the other plots for both PEG-DM and PEG-DA under different concentrations of cellulase are similar.

It can be verified that between the first and second mass measurements, there is a slight decrease of mass for the three hydrogels. However, after that the mass of the hydrogels remains approximately the same, suggesting that PEG is not degraded by cellulase, as expected.

The decrease of mass between the first and second measurements can be explained by the difference of interactions caused by PBS and the solution of cellulase cell culture medium. In the first measurement, before the hydrogels are subject to the presence of cellulase (t=0 h) the pores of the hydrogels are filled with PBS. In the second measurement (t=1.5 h) the PBS in the wells has been replaced with cellulase solution, so now the hydrogel pores are filled with this solution. If there is a decrease in weight but the material is not degraded, then the PBS may cause interactions that make the PEG absorb more water than the cellulase solution.

4.3 Cell viability under cellulase

Preliminary cell viability studies revealed that the cells are sensitive to high concentrations of cellulase (Figure 28). In 1 U/mL cellulase, the cells die after 48 h of exposure to cellulase and at 0.2 or 0.5 U/mL the cells proliferate to confluence.

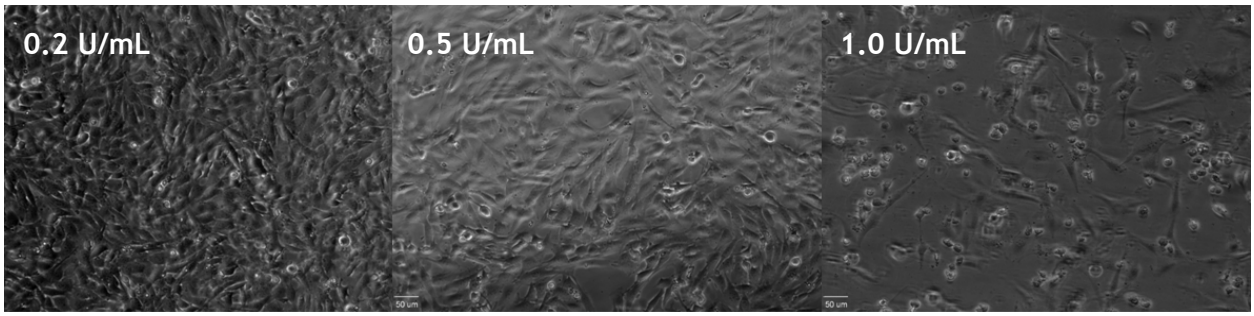

Figure 28. Phase contrast images of Balb/3T3 fibroblasts (50,000 cells/mL) cultured in the presence of cellulase in the cell culture medium at different concentrations. (scale bar – 10 µm)

In the second cell viability study, a live-dead assay was conducted.

After 24 h of culture in cellulase, the cells were imaged under an inverted fluorescent microscope and due to the live-dead assay it was possible to count the number of live and dead cells. Cell viability and cell density for each cellulase were determined. An example of the images obtained is presented in Figure 29. The cells stained with red are dead, and the ones stained with green are alive. The results obtained are presented in Figures 30 and 31.

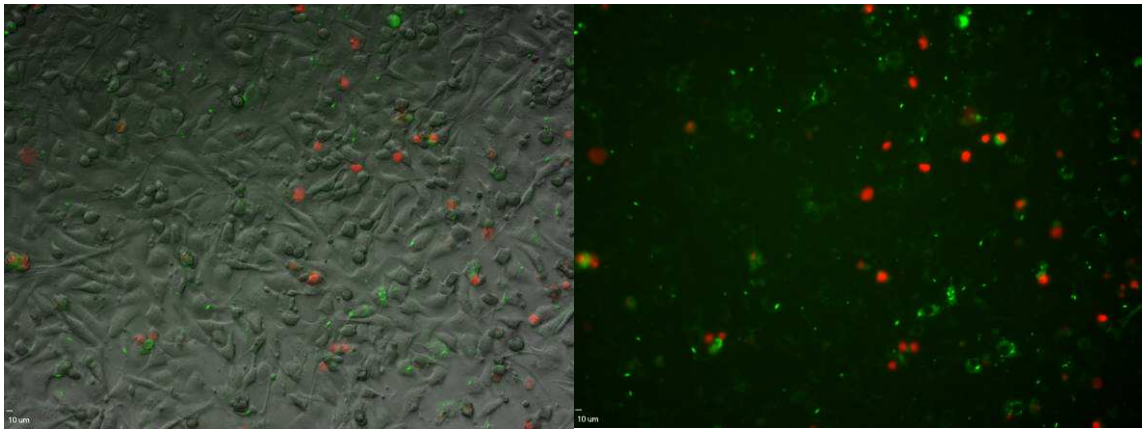

Figure 29. Live-dead assay for a concentration of 0.5 U/mL. (scale bar – 10 µm)

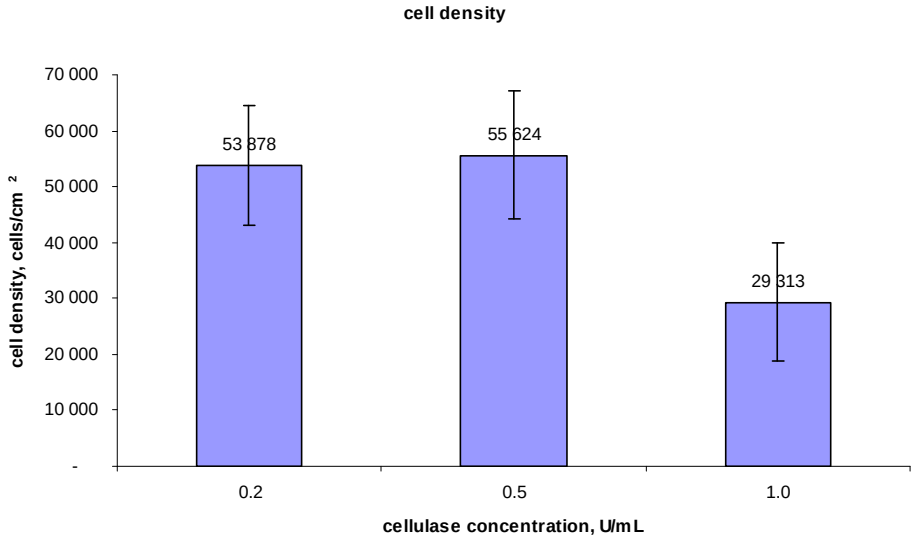

Figure 30. Cell density after 24 h under different concentrations of cellulase.

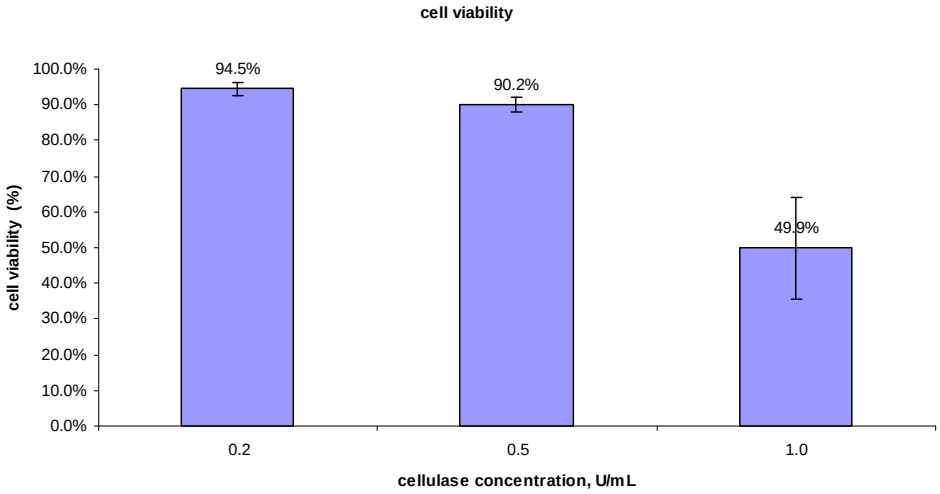

Figure 31. Cell viability after 24 h under different concentrations of cellulase.

There is no significant difference between the cell densities when using 0.2 or 0.5 U/mL of cellulase ($p<0.75$). However, there is a significant difference between the cell densities resulting from exposure to 0.5 or 1.0 U/mL of cellulase ($p<0.0002$). This suggests that the best concentrations of cellulase are 0.2 and 0.5 U/mL.

Regarding cell viability, there is a significant difference between 0.2 and 0.5 U/mL ($p<0.0004$) and also between 0.5 and 1.0 U/mL ($p<1\times10^{-6}$). However, the two lowest concentrations present very similar results. There is a decrease of approximately 4% in cell viability between 0.2 to 0.5 U/mL of cellulase. However, the 0.5 U/mL cellulase solution is

thought to degrade the patterns faster, so a loss of 4% in cell viability will be compensated by a shorter exposure time of the cells to the cellulase solution.

4.4 Modification of glass coverslips

Glass coverslips activated using Method 1A were the first that were sent to Dr. Binil Starly's laboratory, at the University of Oklahoma, School of Industrial Engineering, so that they could use his technique to create the micropatterns.

After crosslinking the micropatterns, when the excess solution was removed with water, the micropatterns immediately detached from the glass. Therefore, subsequent work was focused on optimizing the activation of the glass coverslips.

Based on published findings, changes were made to the initial procedure described by the manufacturer, as described in Method 2A.

The new glass coverslips, activated using the revised procedure, Method 2A, were sent to Dr. Binil Starly. After patterning, the gels detached from the coverslips only after they were immersed in water overnight, demonstrating that the revised procedure is more effective at providing reactive sites for gel attachment.

Further optimization steps sought a procedure that would allow the gel to bind to the glass for at least several days. Both procedures mentioned above were repeated, but this time, the initial cleaning of the coverslips was done using only ethanol, instead of soap (Methods 1B and 2B). Glass coverslips prepared using these new procedures were sent to Dr. Binil Starly and he found that Method 2B was more effective, because it allowed the micropatterns to remain attached to the glass for longer periods of time (overnight as opposed to only 30 min with method 1B). After that, efforts were concentrated on further optimization of this method.

An additional improvement to the procedure was changing the initial cleaning step. Chromic sulphuric acid was investigated to clean the glass coverslips (Method 3).

The two methods of cleaning the coverslips, ethanol (Method 2B) and chromic sulphuric acid (Method 3) were then compared.

A decision was made to test these methods on Dr. Leach's lab before sending them to Dr. Starly. The coverslips were activated using Methods 2B and 3 and PEG hydrogels were crosslinked onto the coverslips. It was possible to use PEG-DM available in Dr. Leach's lab, as well as PEG-DA, which Dr. Starly provided.

After crosslinking the gels on top of the coverslips, different methods to improve the attachment of the gels to the glass were attempted. These methods focused on optimizing

the extent of gel-gel crosslinking and gel-glass bonding by varying washing and UV exposure steps. The gels were rinsed with water and then were let sit for 2 h before being immersing in water (Option A). Another option that was tested was rinsing the gels with water and putting them back under UV light for 2 h before immersing them (Option B). Finally, for Option C, the gels were immersed for 2 h in a solution of tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) after rinsing with water. This solution was used to promote the crosslinking of polymers using a second chemically-induced free-radical reaction.

After these different attempts to improve the attachment of the gel to the glass, the gels were immersed in PBS and monitored until they detached from the glass.

During this experiment some challenges emerged. We used PEG-DM available from our lab, as well PEG-DA provided by Dr. Starly. The solutions prepared with both polymers were 20% (w/v). The PEG-DM was exposed to UV light for about 5-10 min to crosslink. However, it appeared that the PEG-DA solution takes less time to crosslink, so the excessive time under the UV light caused these gels to break. Therefore, it was not possible to take many conclusions from this initial experiment.

While analysing the results obtained with PEG-DM, it appears that the coverslips cleaned with ethanol work better than the ones cleaned with chromic sulphuric acid, however, this experiment was repeated to obtain more exact results.

This time, the PEG-DA solution was left under UV light to crosslink for only 1 min.

After crosslinking the gels, the same three methods of improving the attachment of the gels to the glass were tried after rinsing with water (Options A, B and C). After these different attempts to improve the attachment of the gel to the glass, the gels were immersed in PBS and monitored until they detached from the glass.

The results for PEG-DM were somewhat different from the results for PEG-DA.

For PEG-DM, the gels that were let sit for 2 h (Option A) and the ones that were placed in TEMED/APS solution before immersing in PBS (Option C) remained attached to the coverslips for over a week. The gels that were placed again under UV light (Option B) started to detach after 1 day in PBS.

For PEG-DA, both the gels that were placed under UV light again (Option B) and the ones that were placed in TEMED/APS solution (Option C) broke and came off the coverslips before immersing them in PBS. The gels that were let sit for 2 h remained attached to the coverslips for over a week.

The glass activation method where the coverslips are washed with ethanol (Method 2B) instead of cleaning solution (Method 3) allowed to obtain better results, because on the coverslips prepared through Method 3 the borders of the gels started to detach sooner (after 2-3 days) than on the coverslips prepared through Method 2A.

These results were sent to Dr. Starly and the same experiment was carried out in his laboratory. The same results were obtained there, when using the macro hydrogels. However, they are still having problems with the micropatterns. These continue to detach from the coverslips after a few hours. This may show that the problem with the micropatterns coming off the coverslips might be due to procedures specific to the micropatterning steps, for example, short crosslinking times (6 s) or the small area of contact between the gel and the coverslip when making the micropatterns.

This conclusion allowed coming up with a possible solution. That solution would involve the following steps:

1 - Coat the glass coverslip with a very fine layer of PEG-DA and allow it to crosslink onto the coverslip. This PEG-DA needs to be modified to have sufficient attachment sites for the cells to adhere.

2 - Once this layer is crosslinked it is then possible to crosslink the PEG-DA and CMC-MA micropatterns on top of the thin layer of previously crosslinked PEG-DA.

In the mean time, a new method of glass activation was found in the literature (Method 4), and a test was done using PEG-DM gels (to be able to compare the results with the previous experiments). The gels were let sit for 2 h before immersing them in PBS because in previous tests that was the method that worked better for both PEG-DM and PEG-DA. The new method of activating the coverslips seemed to work just as well as the previous method with these hydrogels (Method 2B). The gels remained attached to the coverslips for over a week. That allowed verifying that this new method does not bring any advantages, regarding the attachment of PEG hydrogels.

This new method was also used in the second experiment of degradation of thin layers of CMC-MA. This method seemed to work better with the 8% gels. However, with the 12% gels there did not seem to be any improvement, for the gels detached almost immediately from the coverslips.

This new method, as well as the method that allowed to obtain better results in the previous studies with PEG (Method 2B), were both tested using CMC-MA hydrogels. For that purpose, CMC-MA hydrogels of different concentrations (8 and 12%) were crosslinked on top of the coverslips. They were let rest for 2 h and were then immersed in PBS. The hydrogels were monitored to verify how long they would remain attached to the coverslips.

The results obtained were somewhat different for the different concentrations of CMC-MA. For the 8% CMC-MA hydrogels, the results obtained for the two different methods were similar. The amount of time that each of the methods allowed the hydrogels to remain attached was the same (over 48 h). For the 12% CMC-MA hydrogels the results were different. The hydrogels crosslinked on top of the coverslips that were activated using Method 4 started to detach after being immersed overnight

in PBS. As for Method 2B, it allowed to obtain similar results as the ones obtained with the 8% hydrogels (over 48 h of attachment).

On the one hand, it was decided to use the 8% CMC-MA solution when working with the patterns, seeing as these hydrogels will be more easily degraded. For that reason, any of the methods would work similarly, as verified for this concentration. On the other and, Method 4 uses a greater concentration of silane, which makes it more expensive. This procedure also requires much more time for the activation of the coverslips. For these reasons, the method that was chosen to be used in future experiments was Method 2B.

4.5 Micropatterns

Dr. Starly sent the first micropatterns that he produced and it was possible to image those under an inverted microscope using phase optics.

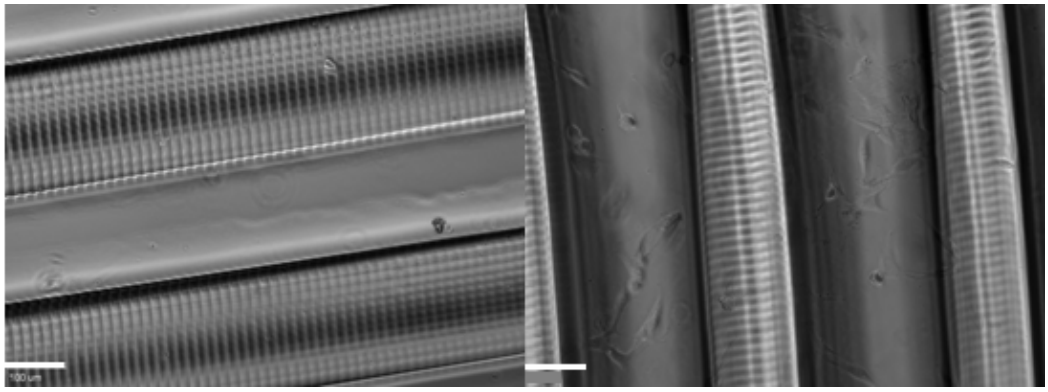

Figure 32. First PEG micropatterns produced by Dr. Starly. (scale bar – 100 μm)

Figure 32 depicts images of the first micropatterns. These micropatterns were created using a mask to make lines on the same order of magnitude in size as the final square patterns we will synthesize for the cell studies. The lines allow us to quickly assess pattern dimensions and cell compatibility. On the picture in the right, it is possible to see the Balb/3T3 fibroblasts that were cultured on the micropatterns, which show that it is possible to culture the cells in the materials.

One fact that is interesting is that the PEG micropatterns show a criss-cross pattern that even Dr. Starly did not know was there. He only realized it after these pictures were sent to him.

After these, more micropatterns from Dr. Starly were received.

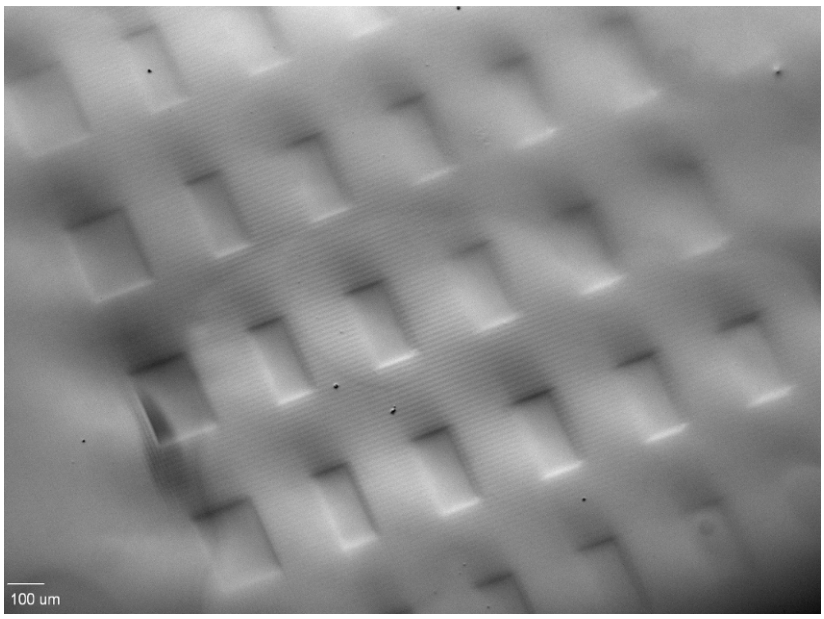

Figure 33. Second PEG micropatterns produced by Dr. Starly. (scale bar – 100 µm)

In Figure 33 it is possible to visualize the most recent micropatterns that Dr. Starly sent. These already have the squares but are still only made of crosslinked PEG-DA. He will add the CMC-MA micropatterns in the near future.

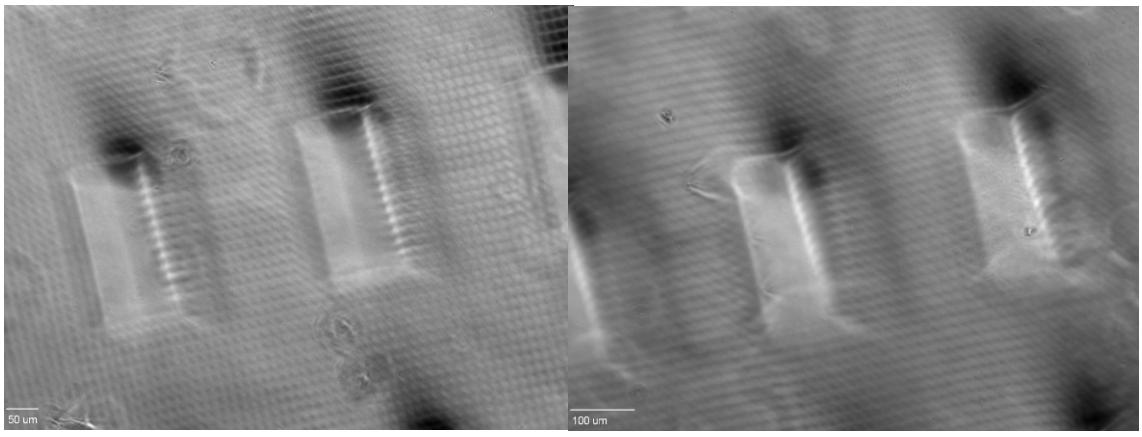

Figure 34. Second PEG micropatterns produced by Dr. Starly. (scale bar – 50 µm on the left; 100 µm on the right)

Once again, in Figure 34 it is possible to see the criss-cross pattern on the PEG micropatterns.

4.6 CMC-MA/PEG-DM patterns

Although the plasma cleaner was not working, the alternative procedure seems to have been successful. Macro patterns on glass slides were produced and were imaged on an inverted fluorescent microscope. On Figure 35 it is possible to see two pictures of the pattern and on Figure 36 it is possible to see the pictures of the two CMC/PEG interfaces obtained in one of the slides.

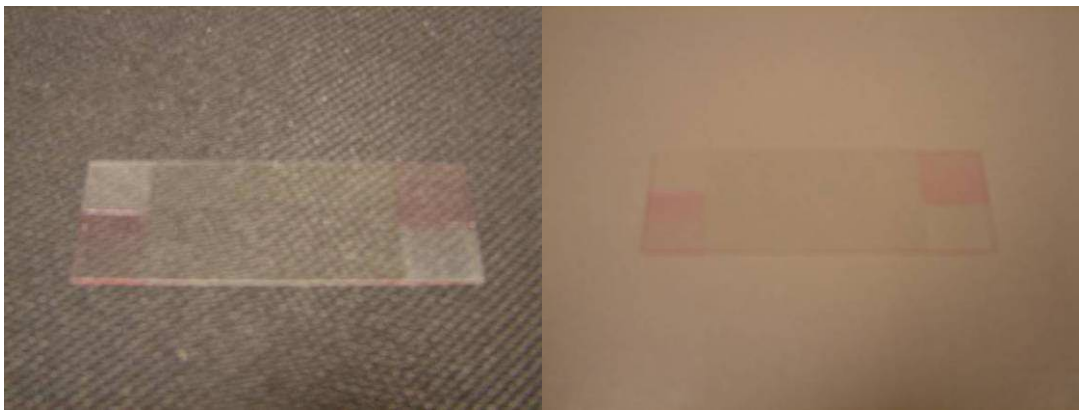

Figure 35. Pictures of the macro CMC/PEG patterns.

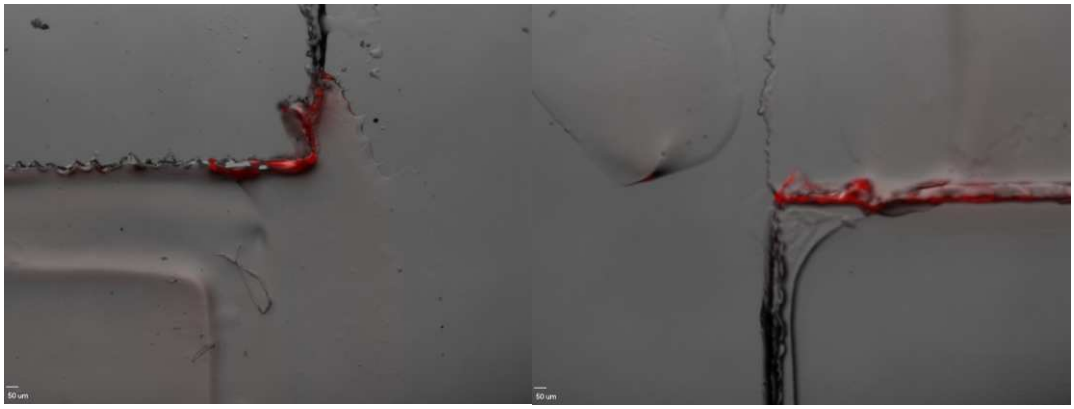

Figure 36. Pictures of both CMC/PEG interfaces on one of the slides. (scale bar – 10 µm)

The next step is to culture 3T3 fibroblasts on the slides, degrade the CMC-MA with a 0.5 U/mL solution of cellulase, and culture PC12 cells.

During the first cell patterning experiment, the first type of cells, 3T3 fibroblasts, were allowed to adhere overnight (for 9 h). The next morning, medium containing cellulase was added to samples to degrade the CMC-MA. Every 1-2 h this solution was changed for 12 h. At the end of the day, PC12 cells were cultured on the slides. These cells were allowed to

adhere overnight (for 12 h). This corresponds to a total time of 33 h while the patterned slides were immersed in liquid. In the next morning the cells were going to be fixed and imaged on an inverted microscope. However, by that time the PEG-DM pattern had detached from the slide, and the cells had adhered to the entire area of the slide.

For this reason, the experiment was repeated with decreased incubation steps, so that the slides would be immersed in liquid for a shorter period of time. For the second experiment, the 3T3 fibroblasts were labelled with $DiOC_{18}$ (green) during the night. They were cultured in the next morning and allowed to adhere and proliferate for 6 h. In this experiment the concentration of fibroblasts was increased to 80 000 cells/mL, because there did not seem enough cells in the first experiment to occupy all the area of the slide. After that, the slides were incubated in medium containing cellulase for 8 h, by the end of which the CMC-MA was completely degraded. At the end of the day, the PC12 cells were cultured and allowed to adhere and proliferate overnight for 10 h. In the next morning the PEG-DM had detached from one of the slides, but was still attached to the other slide. On that slide, the cells were fixed, labelled with DAPI (blue) and imaged under a fluorescent inverted microscope. Fixing and labelling the cells with DAPI takes about an hour, so this time, the period of time in which the slides were immersed in liquid was only of 25 h.

In figure 37 it is possible to see the cells that attached to the site where the CMC-MA had been patterned. This proves that the CMC-MA was already completely degraded by the time the PC12 cells were cultured.

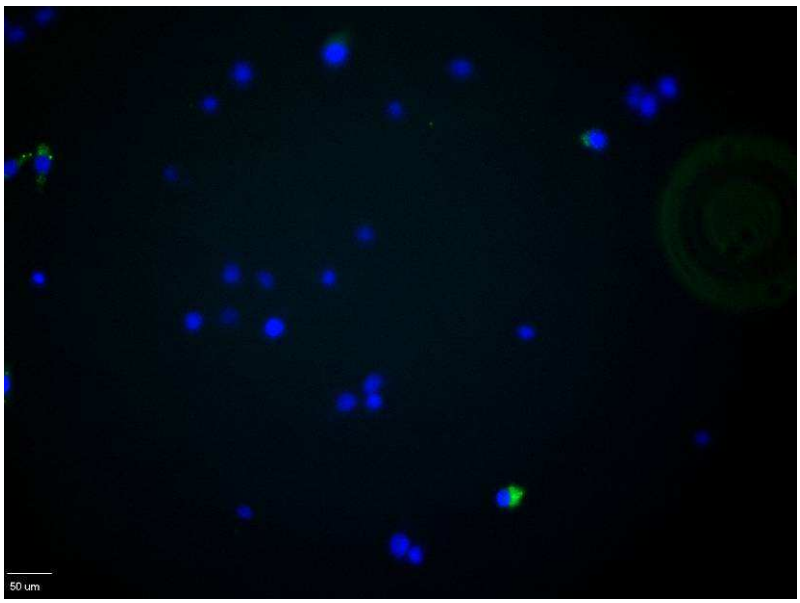

Figure 37. Cells attached to the site where the CMC-MA had been patterned. (scale bar – 50 µm)

This experiment was repeated once again, and this time, the only step that was possible to shorten even more was the PC12 incubation. After the PC12 cells were seeded, they were allowed to adhere for only 2 h. This corresponds to a total period of time of 17 h while the slides were immersed in liquid. This time, the PEG-DM on both slides remained attached to the glass. The slides were imaged and the pictures obtained are presented in Figure 38.

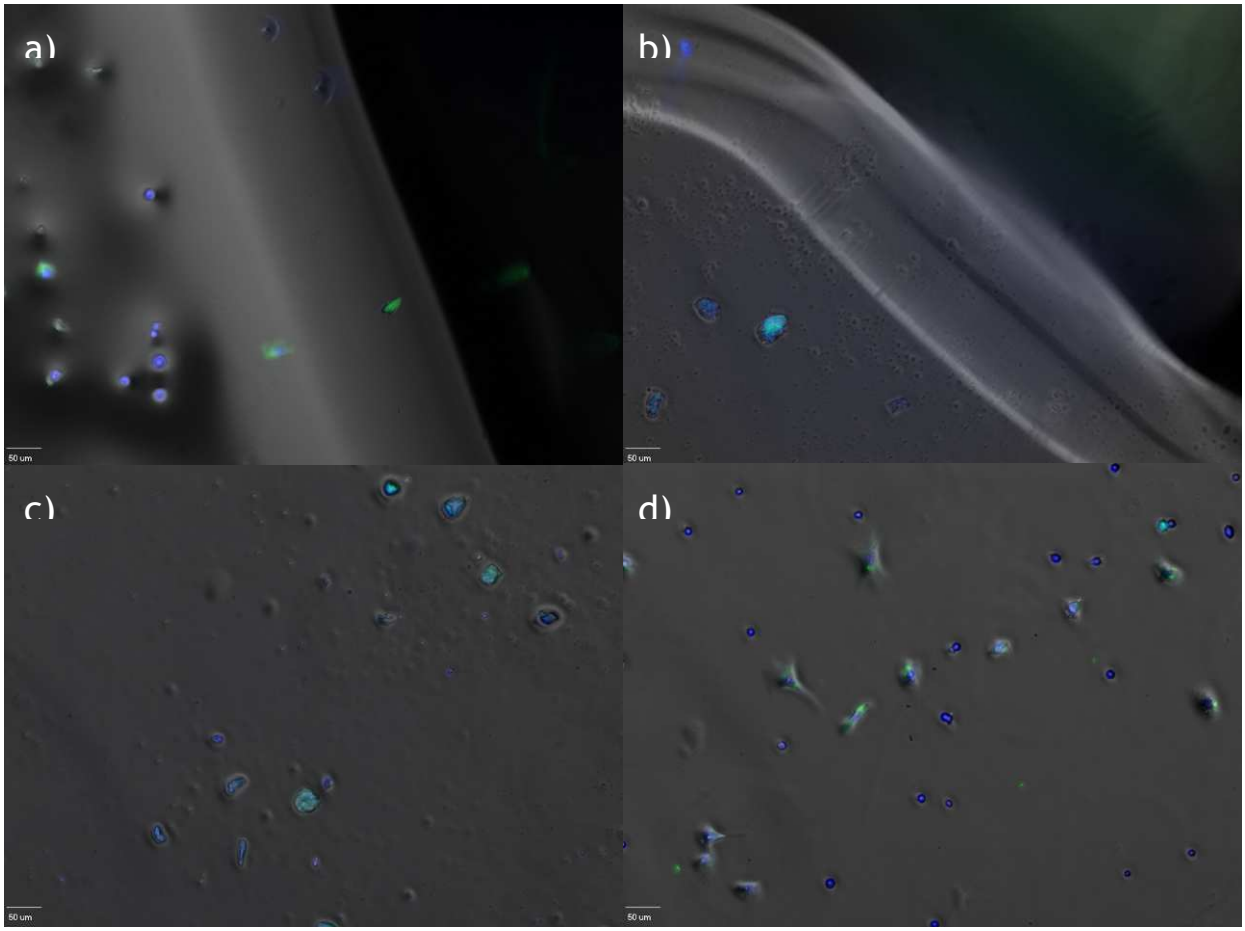

Figure 38. 3T3 fibroblasts (green and blue) and PC12 cells (blue) cultured on patterned slides: a) interface between PEG-DM pattern and the slide; b) interface between PEG-DM pattern and glass where CMC-MA had been patterned; c) area where CMC-MA had been patterned; d) area of free glass. (scale bar – 50 µm)

In Figure 38a it is possible to visualize both fibroblasts and PC 12 cells, as expected. Figure 38b) depicts mostly PC12 cells, although one fibroblast also seems to be present. Figure 38c) depicts mainly PC12 cells, together with some fibroblasts. Finally, in Figure 38d) both fibroblasts and PC 12 cells can be seen, at approximately the same amount.

This experiment, allowed proving that the concept of this project is possible. Although it was not possible to conduct the full experiment with the micropatterns, this shows that once the

micropatterns are produced, it will be possible to culture both types of cells, in a similar way to what was experimented with the patterned slides.

The procedure has been established and the parameters have been optimized for the continued work with the micropatterns.

5 Conclusions

It was possible to take several conclusions from the work conducted during the time of this Project.

From the degradation studies with CMC-MA gels under cellulase, it was possible to conclude that concentrations of 1.0 and 0.5 U/mL of cellulase allow obtaining faster degradation rates.

It was also possible to verify that neither PEG-DM nor PEG-DA hydrogels are degraded by any concentration of cellulase tested (0.2, 0.5 and 1.0 U/mL).

From cell viability studies with 3T3 fibroblasts under the same three concentrations of cellulase, it was possible to come to the conclusion that the highest concentration is harmful to the cells, while concentrations of 0.5 and 0.2 U/mL allow obtaining cell viabilities of approximately 90 and 95%, respectively.

For these reasons, the concentration of cellulase chosen to degrade the CMC-MA on the patterns was 0.5 U/mL.

Between the different methods tested for the modification of glass surfaces with methacrylate groups, the conclusion was that method 2B is the one that allows the gels to remain attached to the glass for longer periods of time.

From cell culture experiments with 3T3 fibroblasts on the micropatterns it was possible to verify the cell compatibility of the materials.

Finally, after optimizing the culture of 3T3 fibroblasts and PC12 cells on patterned slides, it was possible to prove the concept of this project: the co-culture of two different types of cells on PEG/CMC patterns is possible. If the size of the macropatterns and the density of cells to be cultured on them is optimized, it will be possible to culture two different types of cells on different locations of the micropattern.

6 Evaluation of work conducted

6.1 Accomplished Objectives

The main objectives of this Project were:

- **Determine the optimal concentration of cellulase for the degradation of the CMC-MA pattern, through degradation studies of CMC-MA and cell viability studies of 3T3 fibroblasts;** this objective was accomplished. It was possible to determine that the best concentratons for degradation are 0.5 a 1.0 U/mL of cellulase. However, a concentration of 1.0 U/mL causes most of the cells to die, corresponding to a cell viability of only 49.9%. for these reasons, the optimal concentration for the degradation of the CMC-MA patterns is 0.5 U/mL (cell viability of 90.2%).
- **Prove that PEG-DM is not degraded by cellulase;** this objective was also accomplished. Degradation studies with PEG hydrogels under cellulase were conducted and it was verified that the mass of the hydrogels remained the same throughout the experiment, for every concentration of cellulase tested.
- **Verify the cell compatibility of the micropatterns;** once again, this objective was accomplished. 3T3 fibroblasts were successfully cultured on the micropatterns, allowing to verify the cell compatibility of the materials.
- **Optimize the modification of glass surfaces;** from all the methods tested for the modification of glass surfaces, the one that allowed the hydrogels to remain attached to the glass for longer periods of time was chosen for the experiment with the patterns. However, further work may be necessary to improve this step.
- **Culture both cell types (3T3 fibroblasts and PC12 cells) on the micropatterns;** this objective was not accomplished. The micropatterns were not ready in time for this experiment to be conducted during this project. However, the same experiment was conducted on macro PEG/CMC patterns and the procedure is established for when the micropatterns are ready.

6.2 Other work conducted

Other experiments were conducted during this Project. These are presented in detail in Appendix 2.

6.3 Limitations and Future Work

During the timeframe of this Project there was one main limitation that had to do with the collaboration with a professor from another university. Materials had to be sent to him, and from him to our lab. This led to some periods of waiting time. To further complicate this situation, the student that our collaborator had working on the micropatterns went into maternity leave during the month of June. This complicated the work on our part because there was no work being conducted on the micropatterns during that month. This is the explanation to why the last objective was not completed. However, all efforts were employed into conducting the experiment on macro PEG/CMC patterns, and the procedure was established for when the micropatterns are ready.

In the future, Dr. Stary will keep working on the micropatterns. They are now working on his lab on introducing the CMC-MA into the micropatterns. After that is accomplished, the final cell culture experiment in the micropatterns will be possible. It will be necessary to optimize the size of the squares so that they can fit only one cells, as well as the density of cells to be cultured, so that all the squares have one cell in them.

6.4 Final comment

Most of the objectives were accomplished during the timeframe of this Project. The final objective, the only one that was not completed, will probably be accomplished in the near future. The procedure to work with the micropatterns has been established and will have to be conducted before the conferences where this project will be presented.

So, despite the fact that the final objective was no accomplished, all the procedures were established for future work and all the parameters of the final experiment were optimized.

References

- Balgude, A. P., Yu, X., Szymanski, A., Bellamkonda, R. V. Agarose gel stiffness determines rate of DRG neurite extension in 3D cultures. *Biomaterials*, **22**, 1077-1084 (2001)
- Bell, E. Tissue engineering in perspective. In: Lanza R. P., Langer, R., Vacanti, J., editors. Principles of tissue engineering. 2nd ed. San Diego: Academic Press, p. xxxv (2000)
- Bhatia, S. N., Yarmush, M. L., Toner, M. Controlling cell interactions by micropatterning in co-cultures: hepatocytes and 3T3 fibroblasts. *Journal of Biomedical Materials Research*, **34**, 189-199 (1997)
- Bhatia, S. N., Balis, U. J., Yarmush, M. L., Toner, M. Probing heterotypic cell interactions: hepatocyte functions in microfabricated co-cultures. *Journal of Biomaterials Science, Polymer Edition*, **9**, 1137-1160 (1998)
- Bhatia, S. N., Balis, U. J., Yarmush, M. L., Toner, M. Effect of cell-cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells. *FASEB Journal*, **13**, 1883-1900 (1999)
- Bhatia, S. N., Chen, C. S. Tissue engineering at the micro-scale. *Biomedical Microdevices*, **2**, 131-144 (1999)
- Bridgman, P. C., Dave, S., Asnes, C. F., Tullio, A. N., Adelstein, R. S. Myosin IIB is required for growth cone motility. *Journal of Neuroscience*, **21**, 6159-6169 (2001)
- Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M., Ingber, D. E. Geometric control of cell life and death. *Science*, **276**, 1425-1428 (1997)
- Drury, J. L., Mooney, D. J. Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials*, **24**, 4337-4351 (2003)
- Elisseeff, J., Anseth, K., McIntosh, D. S. W., Randolph, M., Langer, R. Transdermal photopolymerization for minimally anvasive implantation. *Proceedings of the National Academy of Sciences USA*, **96**, 3104-3107 (1999)
- Elisseeff, J., McIntosh, W., Fu, K., Blunk, T., Langer, R. Controlled- release of IGF-I and TGF-beta 1 in photopolymerizing hydrogel for cartilage tissue engineering. *Journal of Orthopaedic Research*, **19**, 1098-1104 (2001)

- Flanagan, L.A., Ju, Y. E., Marg, B., Osterfield, M., Janmey, P. A. Neurite branching on deformable substrates. *Neuroreport*, **13**, 2411-2415 (2002)
- Gunn, J. W., Turner, S. D., Mann, B. K. Adhesive and mechanical properties of hydrogels influence neurite extension. *Journal of Biomedical Materials Research*, **72**, 91-97 (2005)
- Hui, E. E., Bhatia, S. N. Microscale control of cell contact and spacing via three-component surface patterning. *Langmuir : The ACS Journal of Surfaces and Colloids*, **23**, 4103-4107 (2007)
- Khetani, S. R., Bhatia, S. N. Engineering tissues for in vitro applications. *Current Opinion in Biotechnology*, **17**, 524-531 (2006)
- Lavik, E., Langer, R. Tissue engineering: current state and perspectives. *Applied Microbiology and Biotechnology*, **65**, 1-8 (2004)
- Leach, J. B., Brown, X. Q., Jacot, J. G., DiMilla, P. A. Neurite outgrowth and branching of PC12 cells on very soft substrates sharply decreases below a threshold of substrate rigidity. *Journal of Neural Engineering*, **4**, 26-34 (2007)
- Lee, C. H., Singla, A., Lee, Y. Biomedical applications of collagen. *International Journal of Pharmaceutics*, **221**, 1-22 (2001)
- Liu, V. A., Bhatia, S. N. Three-dimensional photopatterning of hydrogels containing living cells. *Biomedical Microdevices*, **4**, 257-266 (2002)
- Lu, Y., Mapili, G., Suhali, G., Chen, S., Roy, K. A dgital micro-mirror device-based system for the microfabrication of complex, spatially patterned tissue engineering scaffolds. *Journal of Biomedical Materials Research*, **77**, 396-405 (2006)
- Mann, B. K., Gobin, A. S., Tsai, A. T., Schmedlen, R. H., West, J. L. Smooth muscle cell growth in photopolymerized hydrogels with cell adhesive and proteolytically degradable domains: synthetic ECM analogs for tissue engineering. *Biomaterials*, **22**, 3045-3051 (2001)
- Maruo, S., Ikuta, K. New microstereolithography (Super-IH process) to creat 3D freely movable micromechanism without sacrificial layer technique. From Conference Proceedings: Proceeding of the 1998 international symposium on micromechatronics and human science, p 115-120 (1998)
- Nelson, C. M., Chen, C. S. VE-cadherin simultaneously stimulates and inhibits cell proliferation by altering cytoskeletal structure and tension. *Journal of Cell Science*, **116**, 3571-3581 (2003)

- Nguyen, K. T., West, J. L. Photopolymerizable hydrogels for tissue engineering applications. *Biomaterials*, **23**, 4307-4314 (2002)
- Park J. B., Lakes, R. S. Biomaterials: an introduction, 2nd ed. New York:Plenum Press (1992)
- Pelham, R. J. Jr., Wang, Y. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proceedings of the Natural Academy of Sciences of the United States of America*, **94**, 13661-13665 (1997)
- Pomahac, B., Svensjo, T., Yao, F., Brown, H., Eriksson, E. Tissue engineering of skin. *Critical Reviews in Oral Biology & Medicine*, **9**, 333-344 (1998)
- Sachlos, E., Czernuszka, J. T. Making tissue engineering scaffolds work. Review: the application of solid freeform fabrication technology to the production of tissue engineering scaffolds. *European Cells & Materials Journal*, **5**, 29-40 (2003)
- Schmidt, C. E., Baier, J. M. Acellular vascular tissues: natural biomaterials for tissue repair and tissue engineering. *Biomaterials*, **21**, 2215-2231 (2000)
- Stammen, J. A., Williams, S., Ku, D. N., Guldberg, R. E. Mechanical properties of a novel PVA hydrogel in shear and unconfined compression. *Biomaterials*, **22**, 799-806 (2001)
- Thomas, T. W., DiMilla, P. A. Spreading and motility of human glioblastoma cells on sheets of silicone rubber depend on substratum compliance. *Medical and Biological Engineering and Computing*, **38**, 360-370 (2000)
- Tsang, V. L., Chen, A. A., Cho, L. M., Jadin, K. D., Sah, R. L., DeLong, S., West, J. L., Bhatia, S. N. Fabrication of 3D hepatic tissues by additive photopatterning of cellular hydrogels. *FASEB Journal*, **21**, 790 – 801 (2007)
- Vidic, J., Podgornik, A., Strancar, A. Effect of the glass surface modification on the strength of methacrylate monolith attachment. *Journal of Chromatography*, **1065**, 51-58 (2005).
- Willis, R. K., Skornia, S. L. Effect of collagen gel stiffness on neurite extension. *Journal of Biomaterials Science, Polymer Edition*, **15**, 1521-1531 (2004)
- Yang, S. F., Leong, K. F., Du, Z. H., Chua, C. K. The design of scaffolds for use in tissue engineering. Part 1. Traditional factors. *Tissue Engineering*, **7**, 679-789 (2001)
- Zhang, X., Jiang, X. N., Sun, C. Micro-stereolithography of polymeric and ceramic microstructures. *Sensors and Actuators A*, **77**, 149-156 (1999)

Appendix 1 Degradation of CMC-MA thin layers under cellulase: images

A big number of pictures were taken regarding section 4.1.2 Degradation of CMC-MA thin layers under cellulase. Due to the amount of pictures taken, it was decided to show only some examples in the mentioned section that allow taking the conclusions presented there. However, in this Appendix, all the pictures are shown in the Figures 39 to 58.

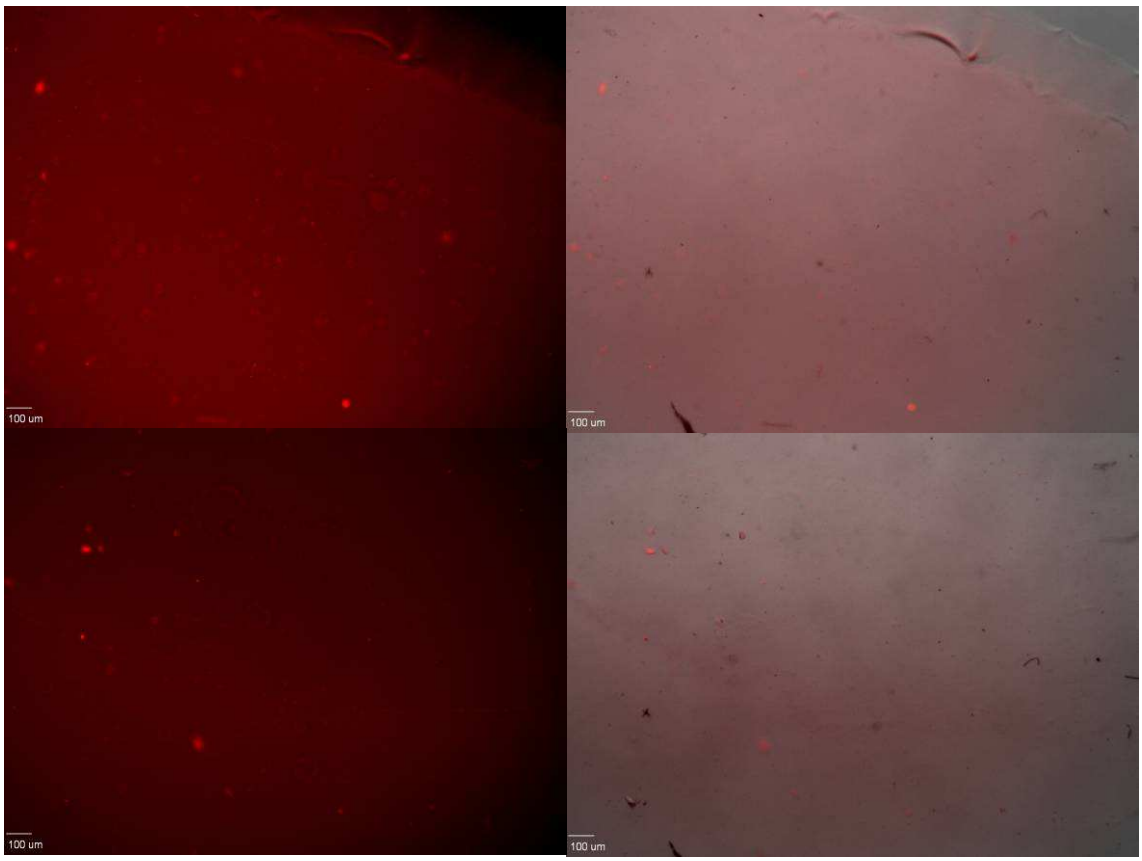

Figure 39: 8% CMC-MA hydrogels before being placed under cellulase. (scale bar – 100 µm)

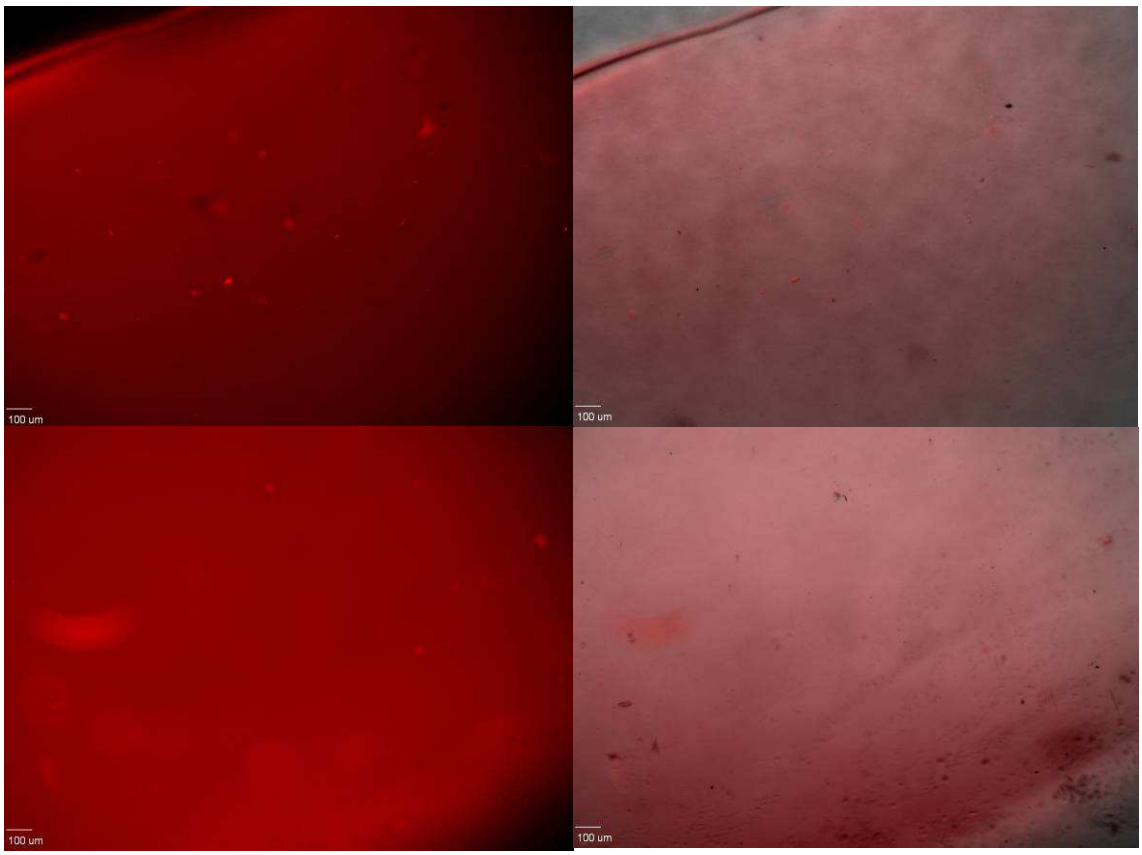

Figure 40: 12% CMC-MA hydrogels before being placed under cellulase. (scale bar – 100 µm)

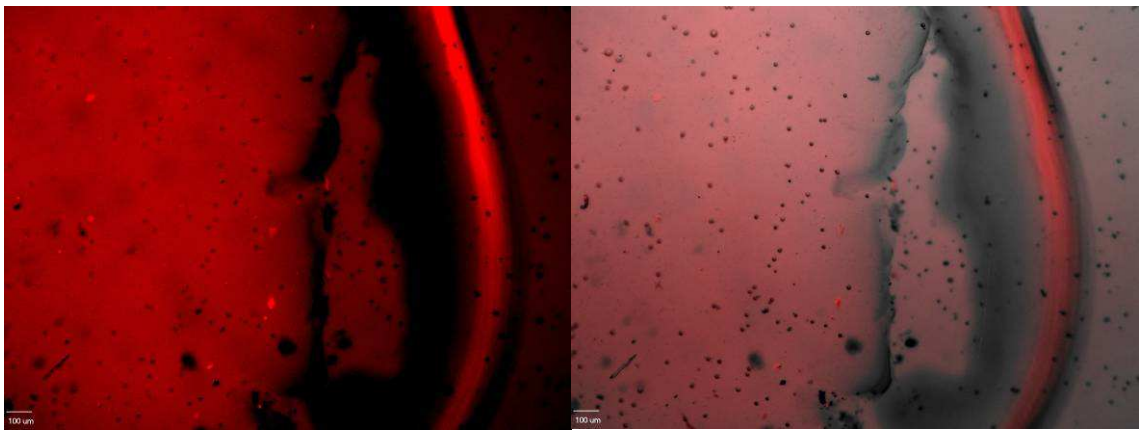

Figure 41. 8% CMC-MA hydrogels after 3 h under 1 U/mL cellulase. (scale bar – 100 µm)

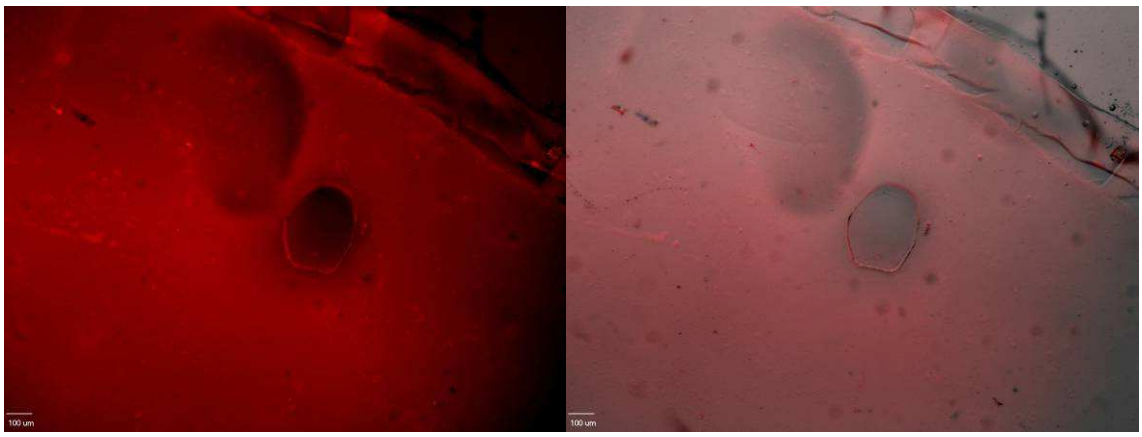

Figure 42. 8% CMC-MA hydrogels after 3 h under 0.5 U/mL cellulase. (scale bar – 100 μm)

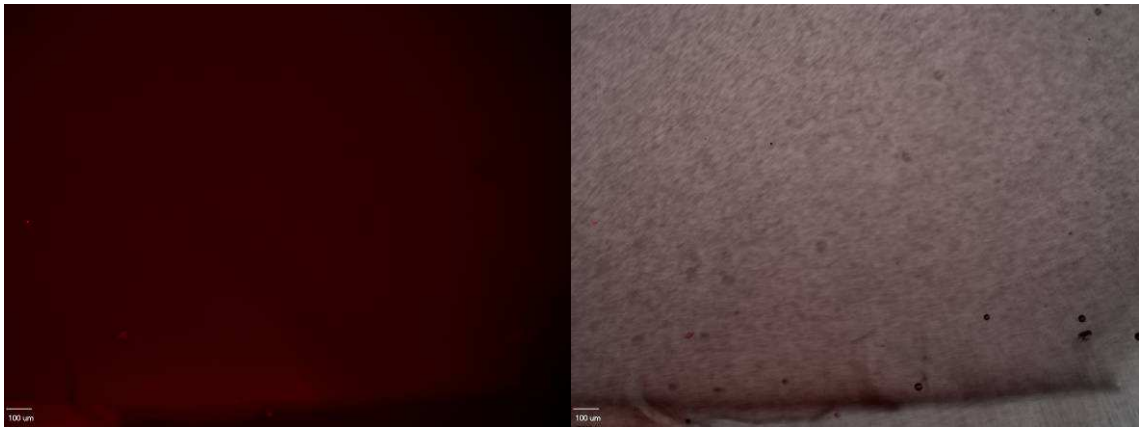

Figure 43. 8% CMC-MA hydrogels after 3 h under 0.2 U/mL cellulase. (scale bar – 100 μm)

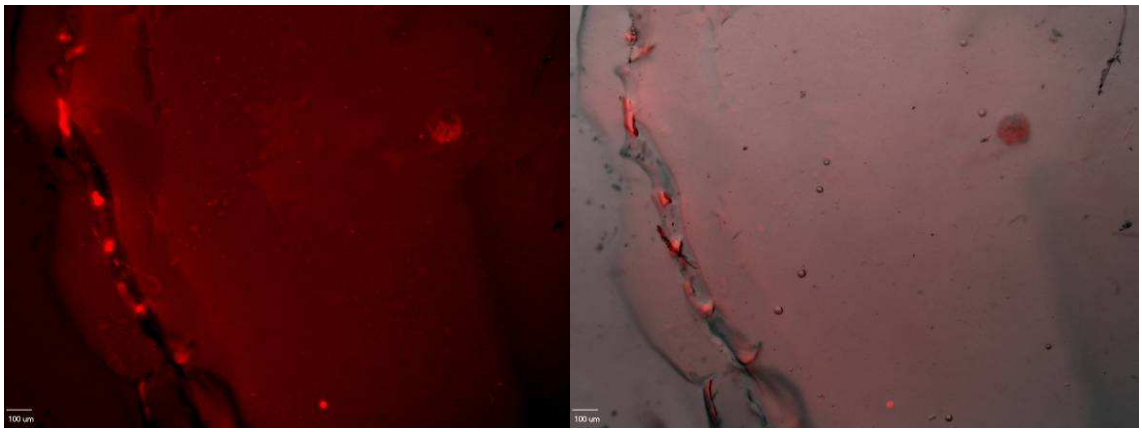

Figure 44. 12% CMC-MA hydrogels after 3 h under 1 U/mL cellulase. (scale bar – 100 μm)

Figure 45. 12% CMC-MA hydrogels after 3 h under 0.5 U/mL cellulase. (scale bar – 100 μm)

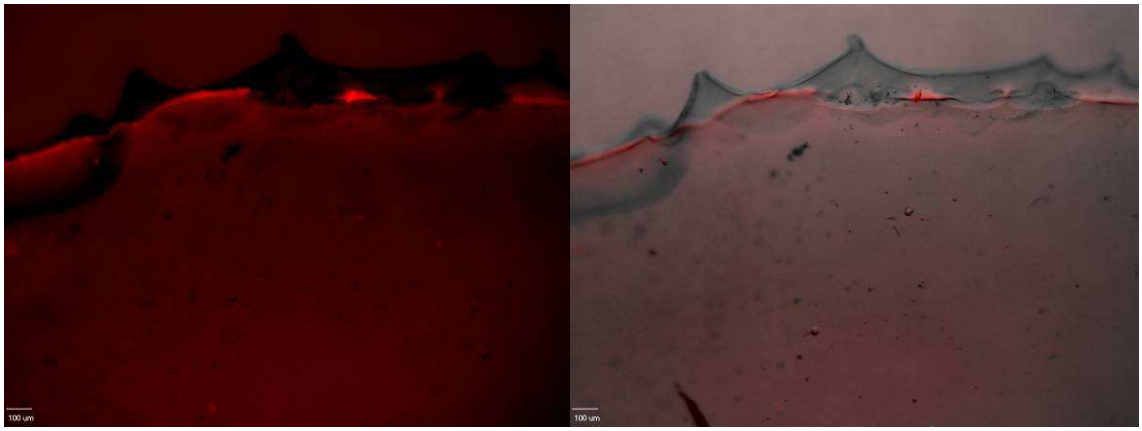

Figure 46. 12% CMC-MA hydrogels after 3 h under 0.2 U/mL cellulase. (scale bar – 100 μm)

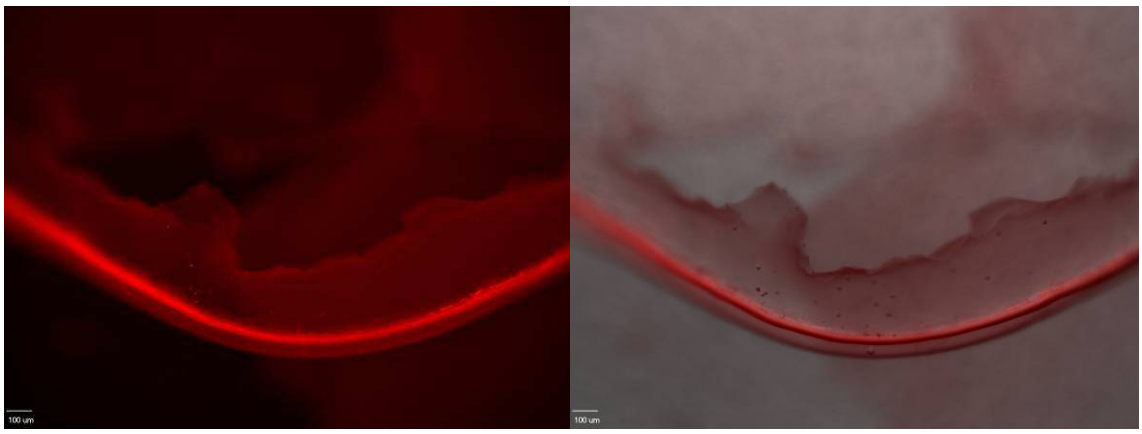

Figure 47. 8% CMC-MA hydrogels after 6 h under 1 U/mL cellulase. (scale bar – 100 μm)

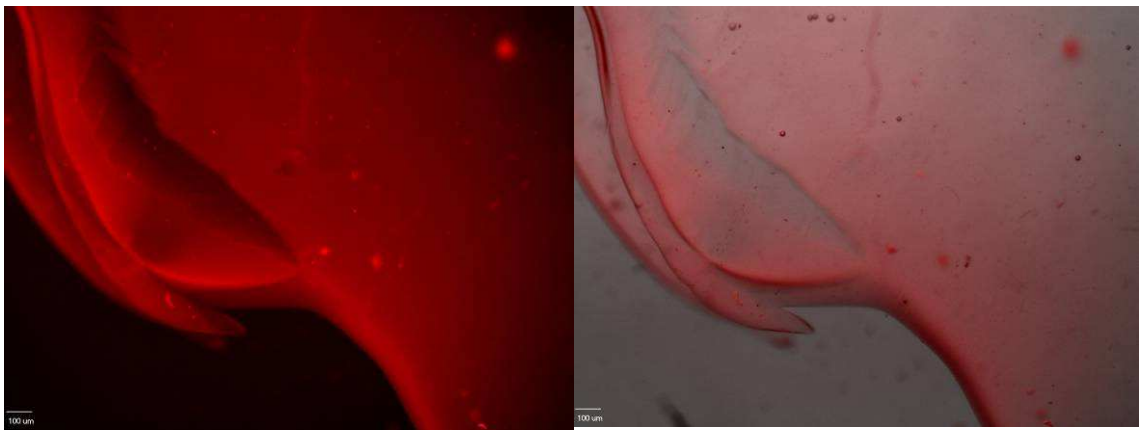

Figure 48. 8% CMC-MA hydrogels after 6 h under 0.5 U/mL cellulase. (scale bar – 100 µm)

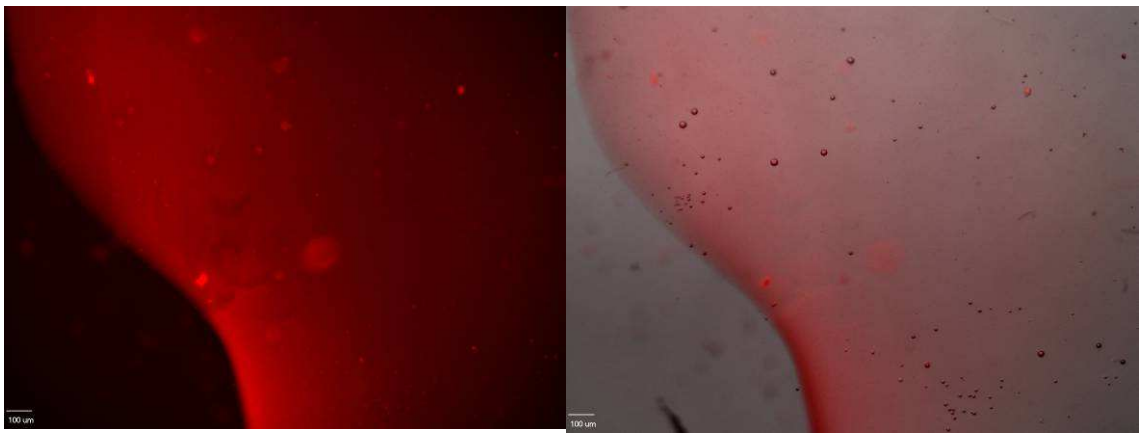

Figure 49. 8% CMC-MA hydrogels after 6 h under 0.2 U/mL cellulase. (scale bar – 100 µm)

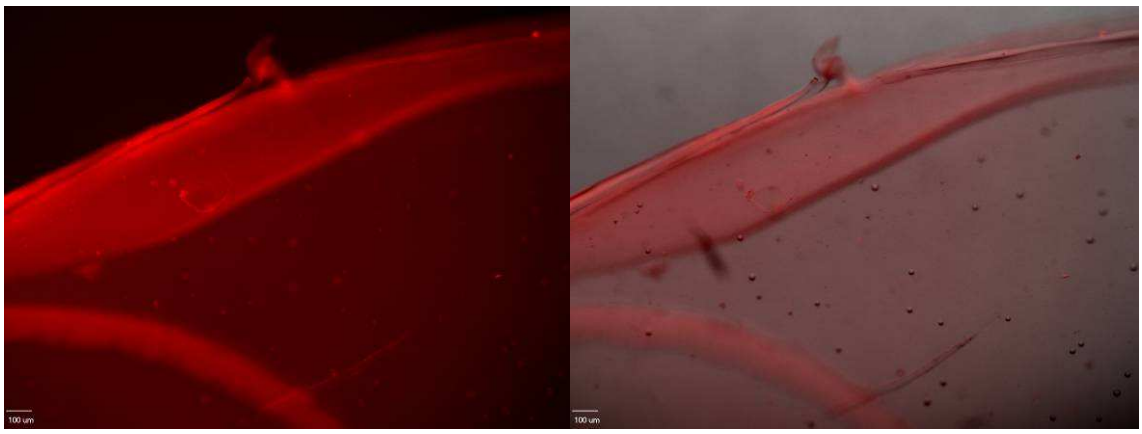

Figure 50. 12% CMC-MA hydrogels after 6 h under 1 U/mL cellulase. (scale bar – 100 µm)

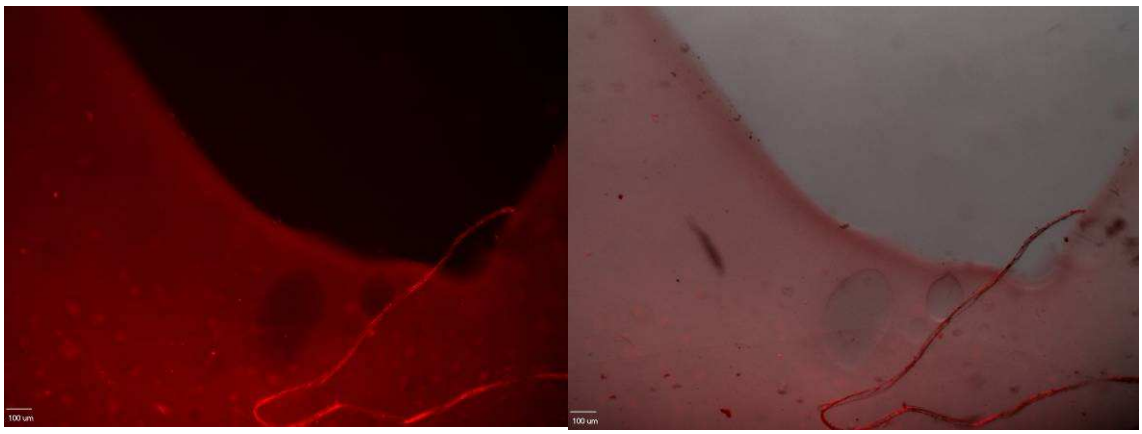

Figure 51. 12% CMC-MA hydrogels after 6 h under 0.5 U/mL cellulase. (scale bar – 100 μm)

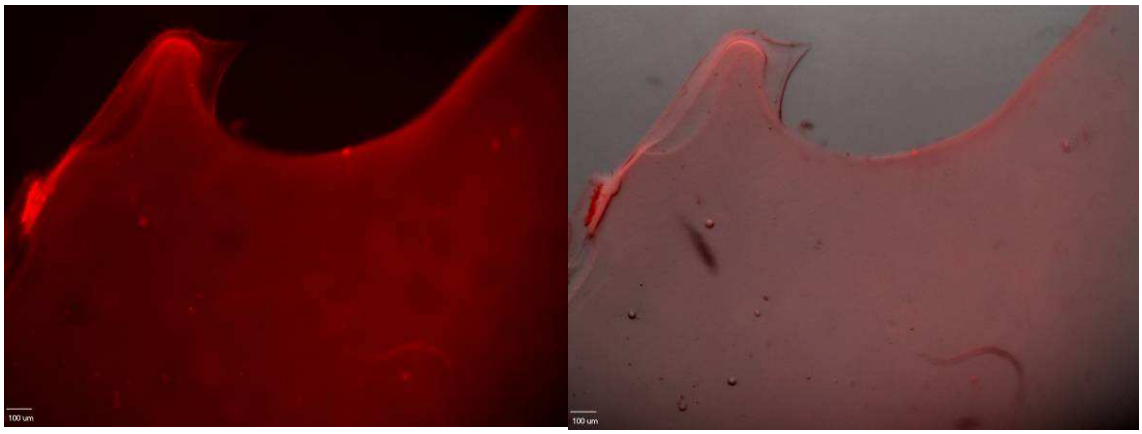

Figure 52. 12% CMC-MA hydrogels after 6 h under 0.2 U/mL cellulase. (scale bar – 100 μm)

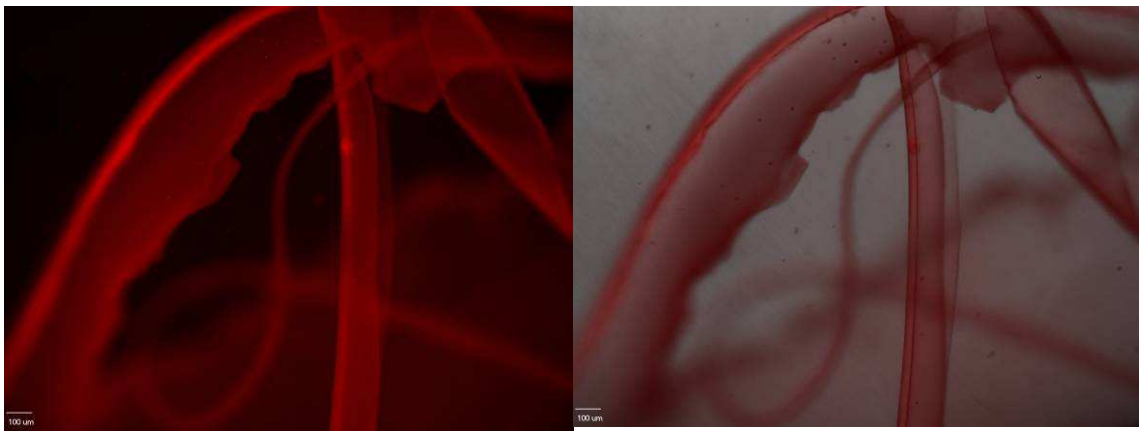

Figure 53. 8% CMC-MA hydrogels after 24 h under 1 U/mL cellulase. (scale bar – 100 μm)

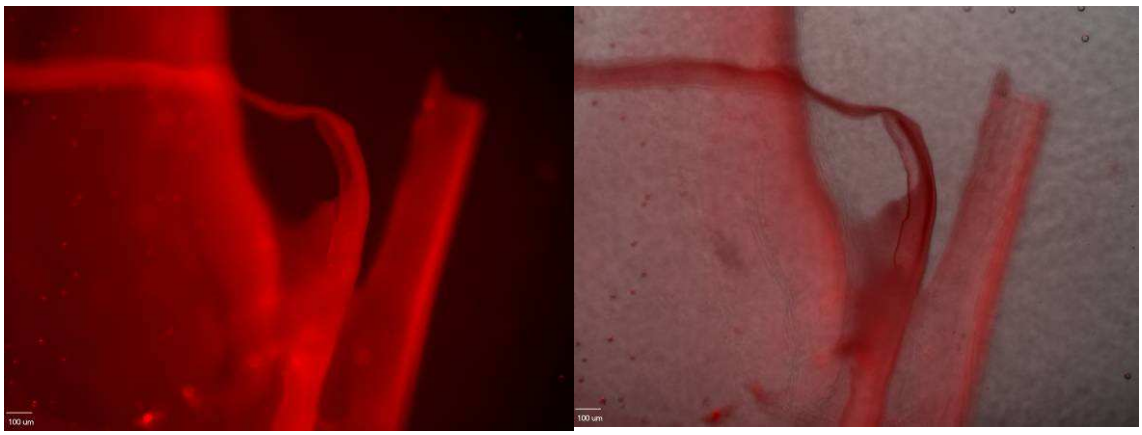

Figure 54. 8% CMC-MA hydrogels after 24 h under 0.5 U/mL cellulase. (scale bar – 100 µm)

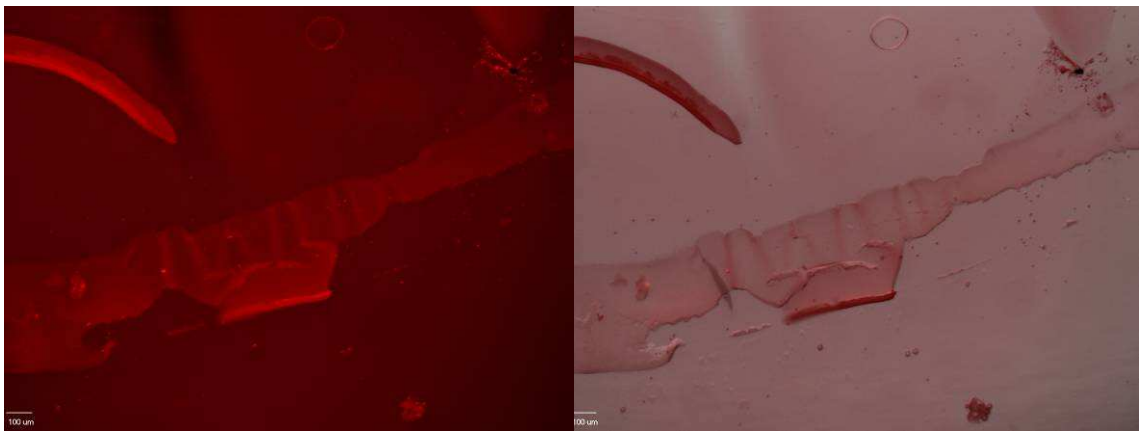

Figure 55. 8% CMC-MA hydrogels after 24 h under 0.2 U/mL cellulase. (scale bar – 100 µm)

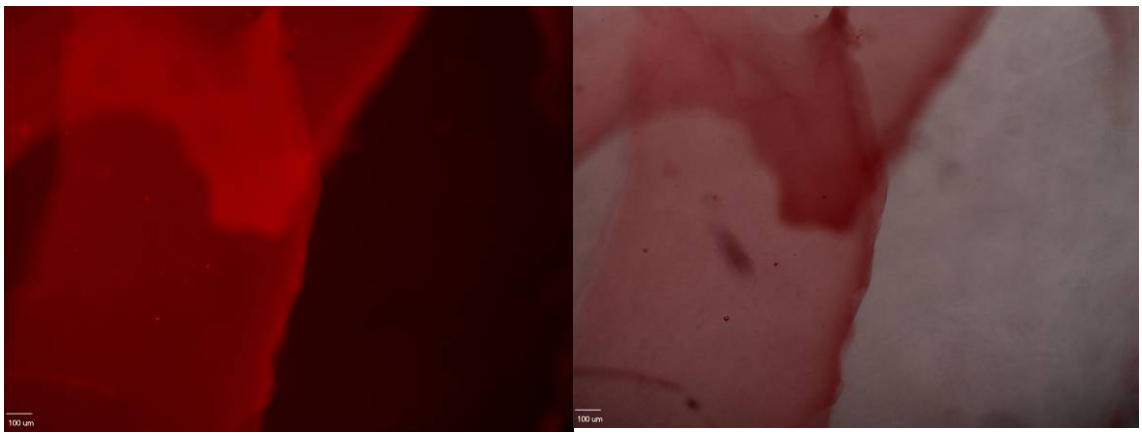

Figure 56. 12% CMC-MA hydrogels after 24 h under 1 U/mL cellulase. (scale bar – 100 µm)

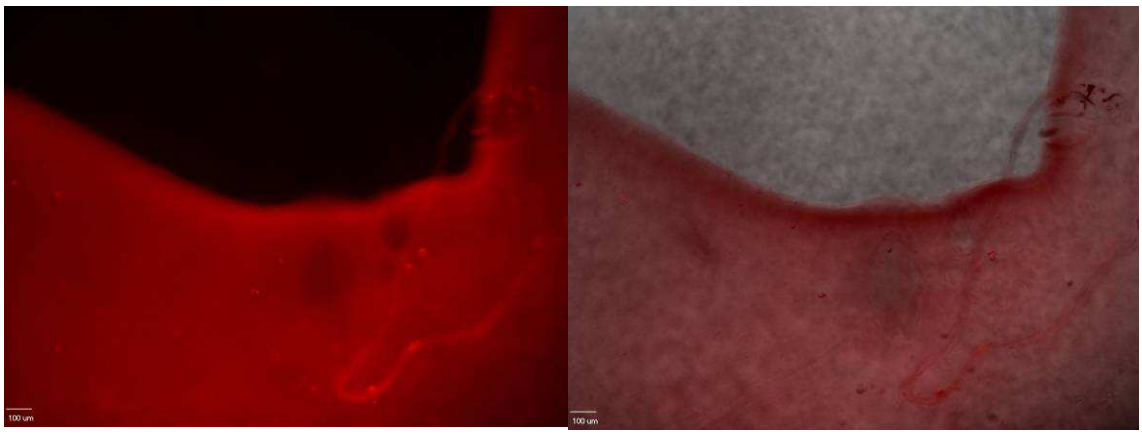

Figure 57. 12% CMC-MA hydrogels after 24 h under 0.5 U/mL cellulase. (scale bar – 100 μm)

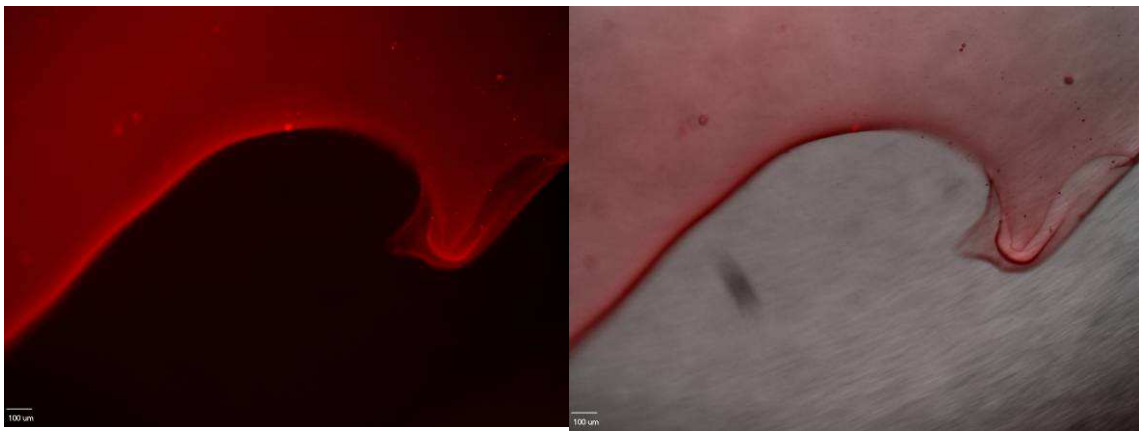

Figure 58. 12% CMC-MA hydrogels after 24 h under 0.2 U/mL cellulase. (scale bar – 100 μm)

Appendix 2 Neural Stem Cells Adhesion and Differentiation in Polyacrylamide Gels

During the time of this project, there where periods when the work load decreased, either due to lack of materials that had been ordered, or due to the interruption of work on Dr. Starly's lab when his student was on maternity leave. For that reason and during those periods, work was done with other materials, polyacrylamide gels. What follows on this appendix is the description of that work, which was not included in the main part of the thesis because it was not possible to take any conclusions in the time that was available.

Rationally designed matrices for nerve tissue engineering and encapsulated cell therapies critically rely on a comprehensive understanding of neural response to both biochemical and biophysical cues (Leach et al., 2007). Biochemical cues are established mediators of neuronal behaviour (e. g., outgrowth). However, biophysical cues, such as substrate stiffness, have only recently been recognized to influence cell behaviour.

It is now known that cell adhesion, proliferation, migration, differentiation and phenotype are affected by the stiffness of the cell's environment (Leach et al., 2007). Work done by Pelham and Wang (1997) demonstrated that fibroblast spreading and migration are greater on stiffer compared to more compliant polyacrylamide hydrogels. After that, the spread area and motility of myoblasts, osteoblasts, vascular smooth muscle cells and endothelial cells have also been demonstrated to correlate with substrate stiffness (Leach et al., 2007). As a consequence, many researchers have proposed a correlation between matrix stiffness and cellular processes in injury, disease and cancer.

Understanding the dependence of cell behaviour on substrate mechanics is less clear, however, for much softer tissues, such as does of the nervous system.

Two general relationships between neurite behaviour and matrix stiffness have been presented: (1) neurite outgrowth varies inversely with the stiffness of two-dimensional substrates (Flanagan et al., 2002) and three-dimensional hydrogels (Balgude et al., 2001; Willis and Skornia, 2004), and (2) neurite outgrowth occurs at a maximum rate at an intermediate range of stiffness (Gunn et al., 2006) in a manner analogous to the effects of adhesion on motility (Bridgman et al., 2001). These relationships contrast with observations that the spreading and motility of malignant astrocytes increase as a threshold response to increasing substratum stiffness (Thomas and DiMilla, 2000).

With this work it was intend to examine the response of neural stem cells (NSC) to a broad range of substrate stiffness, including values corresponding to physiological nerve tissue and to hydrogels previously utilized in studies of cell response on compliant substrates. The main objective was to conduct a comprehensive analysis of multiple quantitative measures of the behaviour of these cells (e. g., number of cells adhered, percentage of cells that differentiate, area occupied by cells) for a large and statistically significant sample population.

Polyacrylamide hydrogels

The first step for this experiment was to activate the coverslips so that the polyacrylamide gels would remain attached to the glass. For that, coverslips with 25 mm of diameter were run over a Bunsen burner flame and placed on a tray covered with foil and absorbent paper with the flame-activated side up. After this, it was necessary to keep track of the activated side of the coverslips. With a cotton swab, the top of the cooled coverslips were smeared with a 0.1 M solution of NaOH and the coverslips were allowed to dry for approximately 10 min. With another cotton swab the top of the coverslips were smeared with 3-aminopropyltrimethoxy silane and allowed to dry for 5 min.

The coverslips were transferred to 6-well plates and then rinsed three times with deionised water. Fresh deionised water was added to the plates and they were placed on a rocker for 10 min. This step was repeated another two times. After that the coverslips were rinsed two more times with deionised water.

3 mL of a 0.8% solution of gluteraldehyde in PBS was added to each well and the plates were placed again on a rocker for 30 min. The gluteraldehyde solution was removed and the coverslips were rinsed three times with deionised water.

The coverslips were stored at 4 ºC in deionised water. This way the coverslips will be stable for a few weeks.

For the production of the polyacrylamide gels, some activated coverslips were allowed to dry. A fresh solution of 100 mg/mL of APS in deionised water was prepared, as well as a fresh solution of 1 mg/mL of acrylic acid N-hydroxysuccinimide in deionised water.

The purpose with this experiment is to make four different kinds of gels, with different stiffness. So 1 mL of prepolymer solution was prepared using different quantities of acrylamide and bis-acrylamide solutions.

Table 3. Content of acrylamide and bis-acrylamide in each of the hydrogels.

Gel	% acryl/% bis	40% Acrylamide	2% Bis-acrylamide
1	3/0.04	75 µL	20 µL
2	8/0.04	200 µL	20 µL
3	10/0.2	250 µL	100 µL
4	14/0.7	350 µL	350 µL

For each kind of gel, the quantities mentioned in Table 1 were added to a 2 mL centrifuge tube. After that, another 100 µL of PBS solution and 2 µL of TEMED were added to the tube. The total volume was adjusted to approximately 700 µL with deionised water and the pH of the solution was adjusted to 7.4 ± 0.3 with a few drops of 1 M HCl.

Finally, a solution of fibronectin was added so that the final concentration of fibronectin in the prepolymer solution was 0.2 mg/mL in the softest gel (gel 1) and 0.1 mg/mL in the rest of the gels. 10 µL of the 1 mg/mL solution of acrylic acid N-hydroxysuccinimide in deionised water previously prepared were added, as well as 5 µL of the 100 mg/mL solution of APS in deionised water.

The total volume of the solution was adjusted to 1 mL with deionised water. 50 µL of prepolymer solution were placed on top of each activated coverslip. The solution was covered with an 18 mm diameter unmodified coverslip and stored in the dark for 1 h to allow polymerization.

After that, the top coverslip was carefully peeled and any unreacted acrylic acid N-hydroxysuccinimide was quenched by storing the substrates in a 2 M solution of glycine in PBS overnight at room temperature.

Polyacrylamide hydrogels cultured with DRG's

At first, the hydrogels were cultured with Dorsal Root Ganglion cells (DRG'S), to study the neurite outgrouth on these cells.

To prepare the substrates for neurite outgrowth studies, they were washed briefly in sterile PBS and transferred to sterile 6-well tissue culture plates. The substrates were then washed in sterile PBS for 10 min and then in cell culture medium for 45 min at room temperature on a rocker.

The medium was removed and the substrates were seeded with 3.85×10^4 cells per well in fresh cell culture medium. The cells were allowed to adhere and grow for 2 days and after that the medium was carefully removed and 2 mL of pre-warmed 3% solution of glutaraldehyde in PBS were added to cover each substrate. The plates containing the substrates were placed on a rocker for 5

min at room temperature and were then rinsed with PBS and stored in fresh PBS at 4 ºC until analysis.

Finally, the substrates were imaged in an inverted fluorescent microscope and the pictures captured are presented in Figure 59.

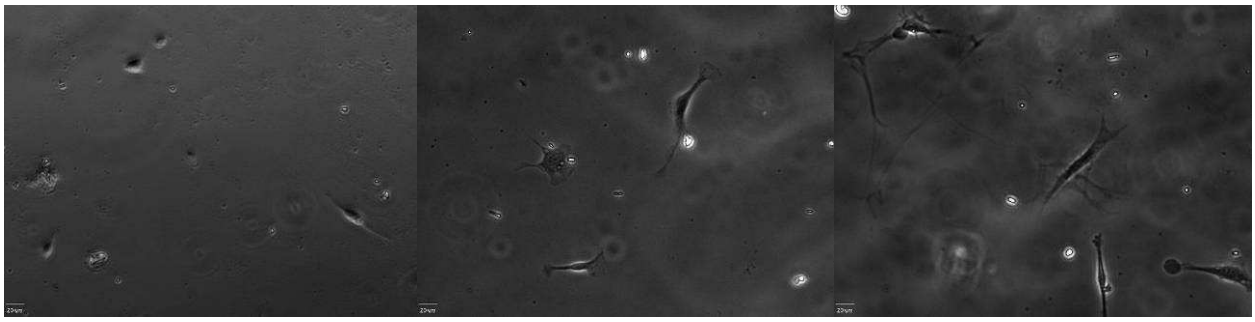

Figure 59. DRG cells cultured in polyacrylamide gels. (scale bar – 20 µm)

However, after this experiment, there were not any more DRG's available, so Neural Stem Cells (NSC) were cultured on the gels during the following experiments.

Polyacrylamide hydrogels cultured with NSC

The gels were prepared in the same way as described before, for the culture of NSC. The cells on the four types of gels were imaged on an inverted microscope. The pictures obtained are presented in Figure 60.

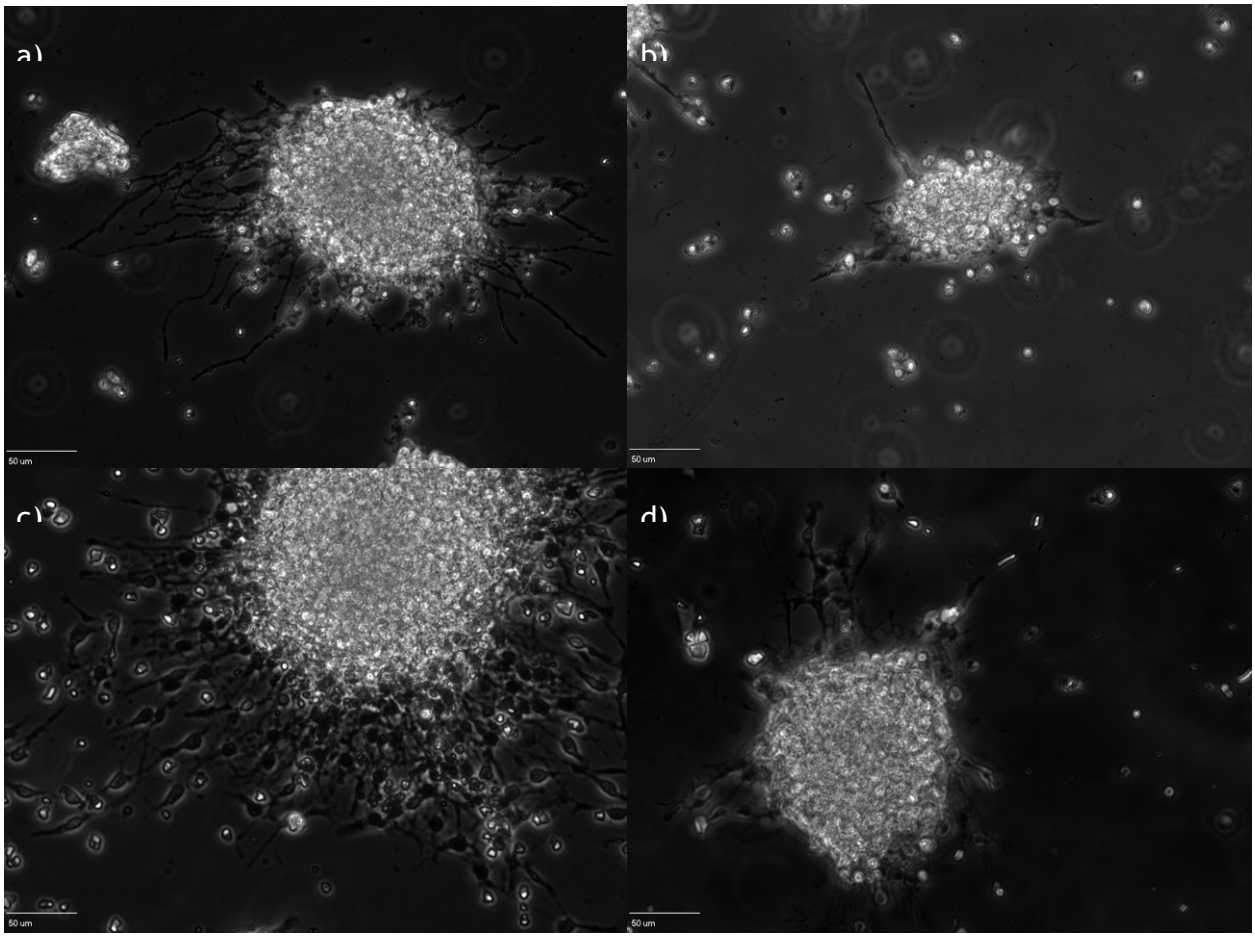

Figure 60. Neural stem cells cultured on on polyacrylamide gels: a) gel # 1 (% acryl/% bis = 3/0.04); b) gel # 2 (% acryl/% bis = 8/0.04); c) gel # 3 (% acryl/% bis = 10/0.2); d) gel # 4 (% acryl/% bis = 14/0.7). (scale bar – 50 µm)

These images only allowed obtaining qualitative results. It seems that the cells adhere more to the stiffest gels (gels 3 and 4). Their size also seems bigger on these gels and they seem to differentiate more.

However, it was attempted to obtain quantitative results by staining the cells on the gels. After staining the cells, the gels were imaged again on an inverted fluorescent microscope. However, very few cells were found after the staining procedure. One possible explanation for this is that during the staining procedure the cells were accidentally removed from the gels. It is convenient that this procedure is conducted more carefully if the experiment is repeated. Some examples of the few images obtained after staining are presented in Figure 61.

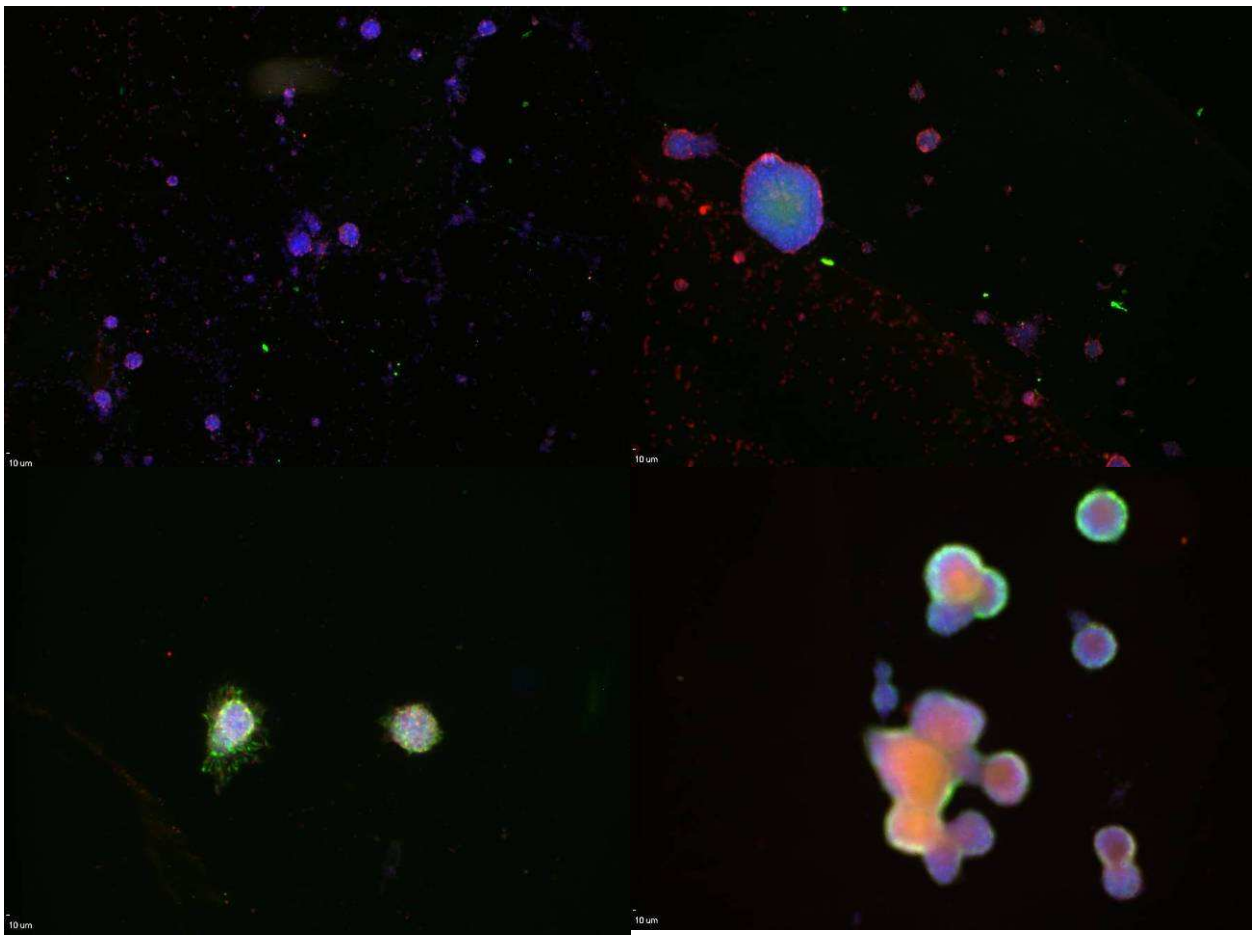

Figure 61. Stained NSC cultured in polyacrylamide hydrogels. (scale bar – 10 μm)

Appendix 3 Cell culture and staining

In this section the procedures for cell culture of different cells are presented, as well as the procedure for the staining of NSC.

Culture of Balb/3T3 fibroblasts

For the culture of fibroblasts on the glass slides, the cells started by being labelled with $DiOC_{18}$. For that a solution of this dye was prepared. 1mL of dye solution is needed for every 500 000 cells. The cells that are going to be cultured are 80 000 cells/ml and 12 mL are needed. That corresponds to a total of almost 2 000 000 cells (1 920 000 cells). So the volume of dye solution needed is 4 mL. The dye solution is prepared by adding 2 µL of dye to each millilitre of cell culture media. So in this case, 8 µL of dye was added to 4 mL of cell culture media.

To label the cells, they were cultured overnight in this solution. To do that, the cells were collected from the plate where they were. To collect the cells the media covering them was dumped from the plate. The plate was then washed with PBS and trypsin was added to the plate. The cells then were incubated for 5 min and after that the plate was washed three times with cell culture media to a 50 mL centrifuge tube.

The concentration of cells in the 50 mL centrifuge tube was calculated using a hemocytometer. For example, in one of the experiments with the glass slides, the concentration of cells in the 50 mL centrifuge tube was 1 010 000 cells/mL. Knowing the amount of cells needed for the experiment was approximately 2 000 000 cells, the volume that had to be used from the 50 mL centrifuge tube was 1.98 mL. This volume was removed and placed in a 15 mL centrifuge tube. All the cell culture media was removed by centrifugation and the solution of dye was added to the cells. The cells were then incubated with the dye solution on a 35 mm tissue culture plate overnight and covered with foil.

In the next morning the cells were collected again in the same way as described before and were placed in 24 mL of cell culture media, to be distributed between the two 10 cm tissue culture plates with the slides.

Culture of PC 12 cells

After the CMC-MA patterns were degraded, PC12 cells were cultured on the slides. To do that, the cells were collected from the plate where they were. The media was removed and the plate was washed with PBS. Trypsin was added to the plate and the cells were incubated for 5 min. After that 10 mL of cell culture media were added to the plate and the cells were gently scraped (with a cell scraper). The cells were collected to a 15 mL centrifuge tube and the media was removed by centrifugation. 10 mL of fresh media were added and the cell clumps were dissociated using a 2 mL syringe. The concentration of cells was calculated once again using a hemocytometer and the volume of media with cells needed was determined. That volume was removed to a 50 mL centrifuge tube and more cell culture media was added until 24 mL, which were distributed between the two plates with the slides.

Culture of Neural Stem Cells

To culture the NSC on the polyacrylamide gels, the cells were collected from the plate where they were to a 15 mL centrifuge tube. In this case the cells do not adhere to the plate so there is no need to use trypsin. The cell culture media where the cells were (proliferative media for NSC) was removed by centrifugation and new media was added to the cells (differentiative media for NSC). The cells were transferred to a 50 mL centrifuge tube and more media was added until the volume needed (3 mL for each polyacrylamide gel).

Fixing cells

The cells were fixed before they were imaged, both in the glass slides and in the polyacrylamide gels. To do that, a volume of 8% formaldehyde corresponding to the volume of media where the cells are cultured (12 mL for the glass slides and 3 mL for the polyacrylamide gels) was added to the plates (to make a final concentration of 4% of formaldehyde). For 10 min the reaction was allowed to take place at room temperature. After that all media and formaldehyde were removed and the initial volume of PBS was added. The cells can be stored in the fridge at 4 ºC.

Label of cells with DAPI

In the end of the experiments with the glass slides, the cells were labelled with DAPI (blue). When this procedure is conducted the cells are already fixed, so the plates are filled with PBS. To label the cells with DAPI the PBS was removed from the plates. DAPI was added to the cells (just enough to cover all the cell surface). The reaction took place for 20 min and the

DAPI was washed with PBS. Finally PBS was added to the plates again and the cells were imaged.

Staining of Neural Stem Cells

The NSC on the polyacrylamide hydrogels were stained following a fluorescent immunocytochemistry protocol.

The coverslips were removed from the wells and placed on 6-well plate lids. They were washed three times for 5 min with PBS. Then a solution of 10% lamb serum diluted in PBS was placed on top of the coverslips for 10 min. After that, during the primary antibody step, 350 µL of a solution of the two primary antibodys used were placed on top of the coverslips for 60 min. In that solution one of the primary antibodys, ß3 tubuline was diluted 500 times in water and the other primary antibody, GFAP was diluted 200 times in water, following the supplier's instructions (Invitrogen).

After that, the gels were washed four times for 5 min with Tris Buffered Saline (TBS). Then, during the secondary antibody step, 350 µL of a solution of the two secondary antibodys used were placed on top of the coverslips for 40 min. In this solution, both secondary antibodys (Alexa Fluor 568 and Alexa Fluor 488) were diluted 500 times in water. From this step until the end of the procedure the coverslips should be covered with foil whenever possible, to protect from the light. After that once again the gels were washed four times for 5 min with TBS. Finally the cells were labelled with DAPI. 300 µL of a 300 nM solution of DAPI were placed on top of the coverslips for 20 min. The gels were washed again for 5 min with TBS and the coverslips were mounted on glass slides with fluorescent mounting media.

Appendix 4 Additional information

During this semester there were regular meetings. I had meetings with my advisor, Dr. Jennie B. Leach, once a week, were we would talk about the work I had done during that week, and we would decide what I would do on the next week.

There were also lab group meetings every two weeks, with Dr. Jennie B. Leach and all the students from the group. In those meeting usually one of the students would present their work to Dr. Leach and the other students.

I presented my work twice. The first time was after two months of starting working and the second time was after I had finished the project.

This project regarding the co-culture of cells on PEG and CMC micropatterns will be presented in several conferences, by Dr. Jennie B. Leach and the PhD student Andreia Ribeiro, in the near future:

- 2008 SfB (Society for Biomaterials) Meeting on Translational Research, Buckhead - Atlanta, GA, September 11-13, 2008 (poster, Dr. Jennie B. Leach)
- BMES (Biomedical Engineering society), St Louis, MO, October 1-4, 2008 (oral presentation, Andreia Ribeiro)
- The AIChE (The American Institute of Chemical Engineers) 2008 Annual Meeting, Philadelphia, PA, November 16-21, 2008 (oral presentation, Andreia Ribeiro).