

Development of a micro-patterned hydrogel scaffold to guide photoreceptor polarization for treatment of macular degeneration

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Declaration of originality

This is to declare that:

- I. All the work included in this thesis was performed by me, unless otherwise stated.
- II. Any collaborators who performed experiments or data analysis have been acknowledged, either at the beginning of each the chapter, or in the corresponding section.
- III. All the publications that have provided reference material for the development of this thesis have been listed in the references section.
- IV. The work reported in this thesis was performed at Imperial College London (UK), University College London (UK), and King's College London (UK).

Raya El Laham

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Abstract

One of the main causes of retinal degenerative diseases is irreversible loss of photoreceptors, which results in visual impairment and can lead to blindness. Current treatment options for photoreceptor degenerative diseases are limited, with most treatments focused on halting the progression of the disease and improving the quality of life of patients. The treatments that do provide some restoration of vision cannot provide the necessary sensitivity and resolution. Photoreceptor replacement therapies aim to deliver healthy photoreceptor cells to the damaged retina to replace those lost through degeneration. Recent studies have demonstrated that using biomaterial scaffolds to deliver photoreceptors can increase the survival, proliferation, integration, and migration of the transplanted cells in the host retina. Furthermore, patterned scaffolds have a demonstrated ability to polarize photoreceptors *in vitro* and improve the likelihood of forming synaptic connection with the host cell once transplanted, and thus increasing the possibility of restoring vision.

The main objective of this thesis was to develop a micro-patterned hydrogel scaffold with a defined geometry aimed at guiding the polarisation of photoreceptors. The hydrogel material was characterized to study the swelling, rheological, diffusion and degradation behaviours of the material, and to identify the optimal polymer concentration that produces the required physical properties. A method for fabricating the patterned scaffold was optimized to be well defined and reproducible. Using a high resolution two photon polymerisation printer, a highly defined negative print with columnar structures was fabricated. Extensive optimisation of the scaffold fabrication process was performed to successfully and repeatably produce the final scaffold in a semi-automated manner. Preliminary cell viability experiments were conducted on non-patterned hydrogels to examine the effect of these concentrations on cell survival *in vitro*.

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Table 5.1: Possible combinations of the HAMA and PEGDMA concentrations.

List of abbreviations

AMD = Age related macular degeneration
APC = Anti-allophycocyanin
ATP = Adenosine triphosphate
AVV = Adeno-associated virus
BCA = Bicinchoninic acid
BM = Bruch's membrane
BSA = Bovine serum albumin
CD = Cell adhesion receptor
Crx = Cone-rod homeobox
DLW = Direct laser writing
Dnase = Desoxyribonuclease
DoF = Degree of functionalisation
DoM = Degree of methacrylation
EBSS = Earle's balanced salt solution
ECM = Extra cellular matrix
EDTA = Ethylenediamine tetraacetic acid
ESC = Embryonic stem cell
FACS = Fluorescence activated cell sorting
FDA = Food and drug administration
GCL = Ganglion cell layer
GelMA = Gelatin methacryloyl
GFP = Green fluorescent protein
HA = Hyaluronic acid
HAMA = Hyaluronic acid methacryloyl
HBSS = Hank's balanced salt solution
hESC = Human-derived embryonic stem cell
HMDS = Hexamethyldisilazane
hPSC = Human pluripotent stem cell

ILM = Inner limiting membrane
INL = Inner nuclear layer
IPL = Inner plexiform layer
iPSC = Induced pluripotent stem cell
IRD = Inherited retinal diseases
LAP = Lithium phenyl-2,4,6-trimethylbenzoylphosphinate
LCA = Leber's congenital amaurosis
LVR = Linear viscoelastic region
MACS = Magnetic activated cell sorting
MC = Methylcellulose
mESC = Mouse-derived embryonic stem cell
MMP = Matrix metalloproteinase
NFL = Nerve fibre layer
NMR = Nuclear magnetic resonance
Nrl = Neural retina leucine zipper
OC = Optic cup
OLM = Outer limiting membrane
OMI = Ovomucoid protease inhibitor
ONL = Outer nuclear layer
OPL = Outer plexiform layer
OV = Optic vesicle
PBS = Phosphate buffered saline
PCDM = Photoreceptor chemically defined media
PCL = Poly(epsilon-caprolactone)
PDL = Poly-D-Lysine
PDMS = Polydimethylsiloxane
PE = Phosphatidylethanolamine
PEG = Poly(ethylene glycol)
PEGDMA = Poly(ethylene glycol) dimethacrylate
PFA = Paraformaldehyde

PGS = Poly(glycerol-co-sebacic acid)
PLGA = Poly(lactic-co-glycolic acid)
RNA = Ribonucleic acid
RPC = Retinal progenitor cell
RPE = Retinal pigment epithelium
RPM = Revolutions per minute
RSPC = Retinal stem progenitor cell
SEM = Scanning electron microscope
STGD = Stargardt disease
TMPS = Tetramethylsilylpropanoic acid
TPA = Two photon absorption
TPP = Two photon polymerisation
UV = Ultra violet
VEGF = Vascular endothelial growth factor

Scope of the thesis

This thesis describes the development of a micro-patterned hydrogel scaffold with a defined geometry aimed at guiding the polarisation of photoreceptors. This defined geometry was designed for the purpose of orienting the photoreceptor cells *in vitro* to improve their integration once transplanted, and the likelihood of forming connections with the host cells.

Chapter 1 provides a general literature review of the anatomy of the eye, the diseases affecting the macula and the current treatment strategies. It also provides a review of the published strategies for cell replacement therapy.

Chapter 2 provides a brief review of the materials used in this project and their synthesis methods, in addition to the development of the scaffold geometry and fabrication technique.

Chapter 3 provides a study of the hydrogel characterisation that evaluates the swelling, rheological, diffusion, and degradation behaviours, and their relevance to the hydrogel geometry.

Chapter 4 provides the hydrogel fabrication optimisation study, aiming to make the process reproducible and semi-automated.

Chapter 5 provides some preliminary cell culture experiments using primary photoreceptor cells, including studies on flat hydrogels and cultures with pre-incubated media.

Chapter 6 provides a summary of the results of the previous chapters and includes suggestions for future work.

Chapter 1: Literature review

1.1 Anatomy and physiology of the mammalian eye

1.1.1 An overview of the eye

The mammalian eye is the specialised sensory organ of the visual system (Figure 1.1). It detects light waves and transduces the signal into electrical pulses that are transmitted to the central nervous system.

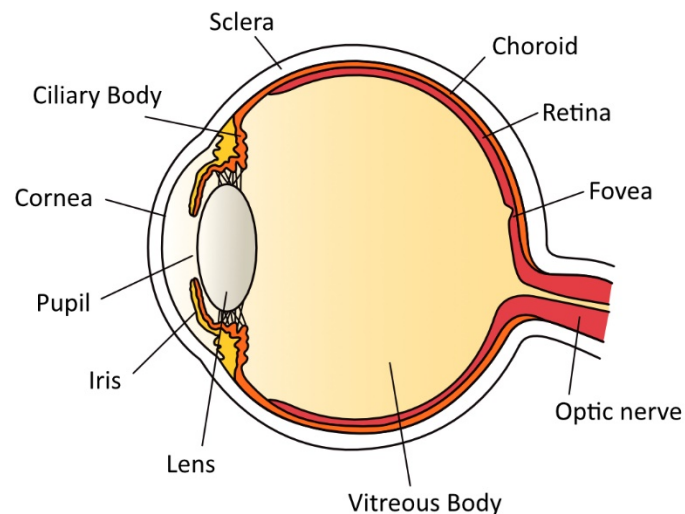


Figure 1.1: Schematic of the anatomy of the mammalian eye. The eye can be divided into three layers, that surround the lens and the fluid components. The corneoscleral layer is the most external layer of the eye and is composed of the cornea and the sclera. The uveal layer is the middle-vascularised layer of the eye and is composed of the iris, the ciliary body, and the choroid. The retina is the innermost sensory layer of the eye and is composed of two layers: the neural retina, which contains the photoreceptors that respond to light, and the retinal pigment epithelium.

The eye can be approximated as a sphere with a diameter of 2.5 cm and a volume of 6.5 mL and is positioned in the anterior portion of the orbit of the skull. The geometry of the eye is a combination of two spheres with varying diameters; the smaller spherical section positioned anteriorly is the cornea, while the sclera constitutes the larger spherical section posteriorly [1].

The eye can be divided into three different layers or coats, often known as tunics. The three coats surround the content of the eye, namely the lens and the fluid components (aqueous humour and vitreous body) [1].

The corneoscleral (or fibrous) layer is the most external layer of the eye and is composed of the cornea and the sclera. The sclera consists of connective tissue, mainly collagen and elastin, and gives the eye its shape. It also protects the inner components of the eye from trauma and gives it its white colour. Furthermore, it provides structural support for the attachment of extraocular muscles to the eye. The cornea is the avascular transparent portion of the eye that allows the light to reach the retina. It is composed of five layers: the corneal epithelium (a thin multicellular epithelium layer), the Bowman's layer (a tough layer composed of collagen type I and laminin), the corneal stroma (a thick layer composed of regularly arranged collagen fibres as well as sparsely distributed keratocytes), the Descemet's membrane (a thin acellular layer composed of collagen type IV), and the corneal endothelium (a squamous monolayer composed of mitochondria rich endothelium cells). The cornea, along with the lens, refract and focus the light onto the retina. Even though the cornea contributes to most of the focusing, its focus is fixed, while the lens can change its geometry to refocus light according to the distance of the object. The cornea and the sclera meet at a region called the limbus [1].

The uveal (or vascular) layer is the middle-vascularised layer of the eye. It lines the corneoscleral layer and is composed of the iris, the ciliary body, and the choroid. The choroid is a pigmented thin layer that contains the blood vessels that supply the retinal cells with oxygen and nutrients, as well as removes the metabolic waste products. It

contains connective tissues and lies between the retina and the sclera. The choroid is divided into four layers. Starting from the furthest away from the retina, the Haller's layer is the outermost layer and consist of the larger blood vessels. Next is the Sattler's layer, which comprises of the medium diameter blood vessels, followed by the choriocapillaris containing the capillaries. Finally, the Bruch's membrane is the innermost layer of the choroid and will be described further in a future section. The ciliary body contains the muscles that control the shape of the lens allowing varying focus for closer or farther objects. It also contains the ciliary epithelium whose role is to produce the aqueous humour, which provides the lens and the cornea with oxygen and nutrients, and removes their metabolic waste, as they are avascular structures. The iris is the coloured area of the area that is seen when looking straight in a person's eye, as it is placed behind the transparent cornea. The pupil is the aperture at the centre of the iris and is controlled by the papillary muscles to regulate the amount of light that can enter the eye. It is black due to the lack of light reflected out of the interior eye [1].

The retina (or nervous tunic) is the innermost sensory layer of the eye and is composed of two layers: the neural retina, which contains the photoreceptors that respond to light, and the retinal pigment epithelium (RPE). As the light is focused onto the retina, the photoreceptors intercept the photons causing a change in the nerve action potentials, and these neural impulses are then transmitted to the brain via the optic nerve, where the information is processed into images allowing vision. The photoreceptor and RPE layers are not connected anatomically, but are adjacent to each other in close proximity, which allows them to support and interact with each other. The layers of the retina will be discussed in further details throughout the rest of this chapter [1].

1.1.2 The embryonic development of the eye

The eye originates from the ectoderm, one of the three germ layers. This layer also differentiates to form the nervous system in the mammal. As illustrated in Figure 1.2, the optic vesicle (OV) is formed by evagination of the neuroectoderm wall around the fourth

week of gestation, and it extends towards the surface ectoderm. The area of the surface ectoderm that contacts the forming optic vesicle develops to become the lens placode. Around the sixth week of gestation, the evagination is followed by invagination of the distal portion of the OV as well as the lens placode which forms a double layered optic cup (OC) and the lens vesicle. With time, the inner layer of the OC develops to become the neural retina, while the outer layer becomes the RPE. The cells bordering the intraretinal space between these two layers differentiates into rods and cones. The lens remains covered by a layer of surface ectoderm that forms the future cornea [2], [3].

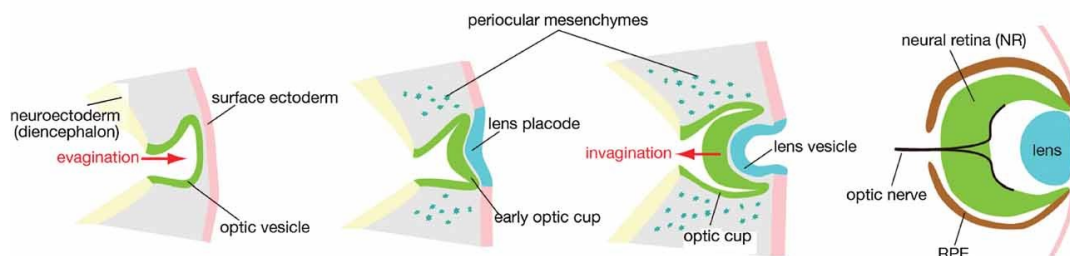


Figure 1.2: Schematic of the development of a mammalian eye. The optic vesicle is formed by evagination of the neuroectoderm wall, followed by invagination of the distal portion of the optic vesicle forming a double layered optic cup and the lens vesicle. With time, the inner layer of the optic cup develops to become the neural retina, while the outer layer becomes the RPE. Figure adapted with permission from [3].

1.1.3 The layers of the retina

The retina is formed of ten layers as shown in Figure 1.3. Starting from the back of the eye, the first is the retinal pigment epithelium or RPE layer, which is a part of the non-neural retina. The RPE basal side lays on the Bruch's membrane, a thin fibrous layer which forms the innermost part of the choroid. The apical side of the RPE cells interact with the second layer made up of the outer and inner segments of the two types of

photoreceptors: rods and cones. The photoreceptors and the RPE cells are closely layered structures that interact together even though they do not form direct connections. The photoreceptors are the light sensing cells of the retina and will be discussed further in this chapter [1].

The next layer is the outer limiting membrane (OLM), which separates the inner and outer segments of the photoreceptors from their nuclei. This is then followed by the outer nuclear layer (ONL), where the cell bodies of the photoreceptors are located. Following this is the outer plexiform layer (OPL) which contains the synapses between the photoreceptor and horizontal cells axon terminals and the dendrites of the bipolar cells, or interneurons [1].

The next layer is the inner nuclear layer (INL) and contains the interneurons and bipolar cells, the amacrine cells, the horizontal cells and the Müller glia cells (not illustrated in Figure 1.3). The bipolar and amacrine cells form synaptic connections with the ganglion cells in the inner plexiform layer (IPL). The Müller glia cells are important components of the retina as they support the neuro-retinal cells, and their processes extend through the entire neural retina. Horizontal cells integrate and regulate the signal from multiple photoreceptors (not already connected to bipolar cells), which allows for increase in visual acuity. The amacrine cells are responsible for modulating the signal that reaches the ganglion cells, whose axons converge to become the optic nerve which transfers the signal to the brain. The electrical signal produced by the photoreceptor is transferred to the bipolar cells (ON and OFF types) and consequently to the ganglion cells, whose cell bodies are in the ganglion cell layer (GCL) and the optic nerve at the nerve fibre layer (NFL). Finally, the inner limiting membrane (ILM) separates the retina from the vitreous cavity [1].

Looking back at these 10 layers, we can see that they are not all “layers”, but rather a single cell layer of RPE cells and three layers of neural cell bodies, as well as their processes and synapses that are between them.

Another important area of the retina is the macula, where it is at its thinnest. It has a diameter of about 5.5 mm in humans and is responsible for providing central vision. At its centre is the fovea, only at 0.6 mm in diameter, composed of closely packed cone type photoreceptors, responsible for the ability to see colour as well as sharp high acuity vision. The remainder of the retina is rich in rod photoreceptors, allowing for scotopic vision. These types of photoreceptors will be studied more closely further along this chapter [1].

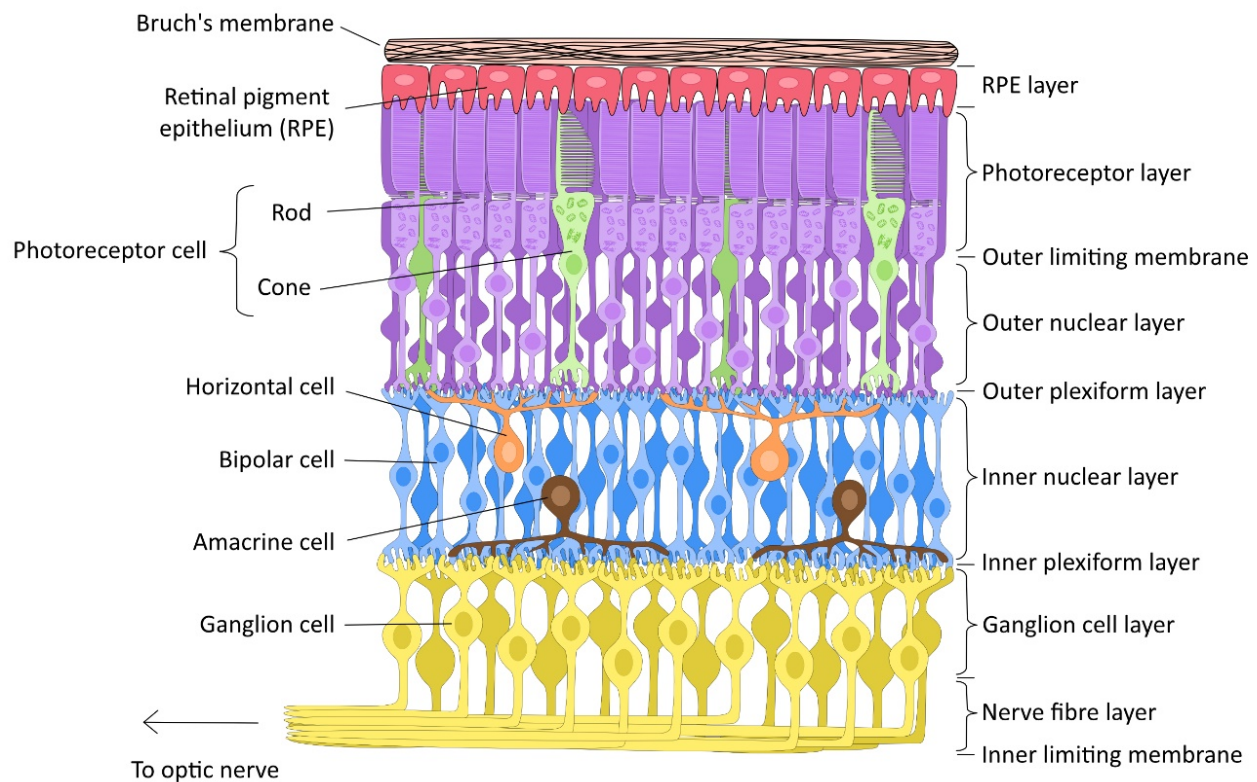


Figure 1.3: Schematic of the layers of the retina. The annotation on the right corresponds to the 10 layers of the retina. The annotation on the left corresponds to the component of the retina.

1.1.4 The Bruch's membrane

The Bruch's membrane (BM) is an acellular sheet of extracellular matrix that separates the retina from the choroid, where the blood vessels are located. The BM plays a crucial role in the function of the retina and cell-cell communication. It controls the passive diffusion of biomolecules between the RPE and the choroid and acts as a filter for the exchange and transfer of these molecules. From the choroid to the RPE, these include nutrients, vitamin A, oxygen, minerals, and lipids, and from the other side, they include carbon dioxide and several waste products, as well as fragments of shed photoreceptor outer segments. Finally, it supports RPE cell adhesion, differentiation, proliferation, and migration [4]. It is made up of 5 layers of collagen and elastin-rich extracellular matrix. Each layer contains different types of collagens, as well as elastin, laminin or glycosaminoglycans. As crucial as the BM is for vision, it also has a role in aging and is also the focal point of age-related macular degeneration, as well as other diseases affecting the eye, which will be explored in section 1.2 [1], [2], [4].

1.1.5 The photoreceptors

There are two types of photoreceptors in the human eye: rods and cones (Figure 1.4). They are located in the second layer of the retina. There are approximately 115 million rods and 6.5 million cones in the human retina, which means the rods outnumber the cones almost 95% to 5% in the eye as a whole. However, the density of the rods and cones varies in different regions of the retina as previously mentioned. The macula is a region in the central part area in the back of the eye just above the optic nerve. It is a cone dominant region, with the fovea only containing cones with a density of 150,000/mm², while the rest of the retina is rod dominant with a density of 30,000/mm². Mice have the same overall ratio of rods and cones, but they lack a macula region [1].

Both rods and cones have a polarised morphology and have the same basic structure but are distinct due to their outer segments that are shaped as they are called. They are formed of several sections and will be described starting from the RPE side. The outer

segment is the part of the photoreceptor that absorbs light. It is formed of voltage gates sodium channels as well as membranous disks (in rods) or infoldings (in cones) that contain a large number of visual pigments. These photo pigments are composed of a protein called opsin and a chromophore called retinal and are responsible for the absorption of the photons and the initiation of phototransduction leading to neuro-electrical impulses. The inner segment contains the mitochondria and has for role to provide adenosine triphosphate (ATP) for the sodium-potassium pump. These two segments are connected through the cilium and connected to the cell body by an outer limiting membrane. The cell body contains the nucleus and organelles. And finally, closest to the visual field is the axons and their terminals (cone pedicle or rod spherule) which release the neurotransmitter glutamate to the bipolar cells and horizontal cells [1].

Rods are responsible for sensing contrast, motion, and brightness, and they are responsible for low intensity light vision or night vision (scotopic conditions). They contain one type of opsin, also called rhodopsin. Cones are responsible for spatial resolution and high acuity vision/perception and colour vision (trichromatic) but require daylight conditions (photopic conditions). There are three types of cone cells that respond to different parts of the visual spectrum. They contain three different opsins that allow them to detect different wavelengths of light, which leads to colour vision. These opsins are distinct in each type of cone: L-cone, S-cone and M-cone type cones are red, blue, and green sensing cones respectively [1].

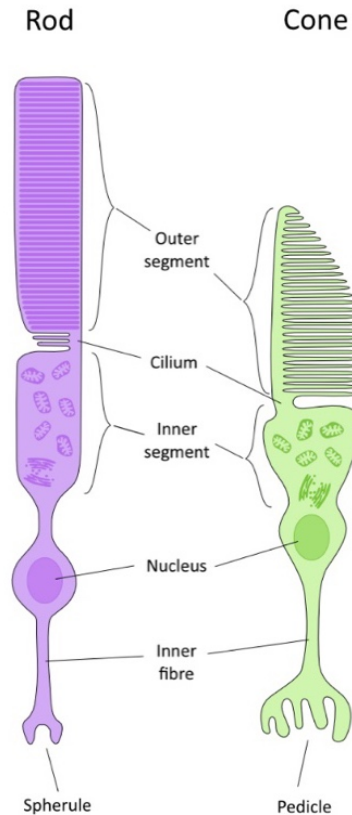


Figure 1.4: Schematic of two types of photoreceptors. Both rods and cones have a polarised morphology and have the same basic structure but are distinct due to their outer segments that are shaped as they are called. Then outer segment is the part of the photoreceptor that absorbs light and is in proximity to the RPE layer.

1.1.6 The retinal pigment epithelium

The retinal pigment epithelium layer is the outermost retinal layer and is made up of a single cell layer of pigmented cuboidal cells. These cells vary in size and shape depending on their location, being more columnar in the centre of the retina and flatter in the periphery. Looking at the cells from the top, they are highly organised hexagonal homogeneous cells. The RPE cells play several roles in the eye, including an essential one in the visual cycle. The pigmentation of the choroid and the RPE cells absorbs the scattered light that passes through the retina and prevents the internal reflection of the

light that enters the eye. The RPE layer also provides a selectively permeable barrier between the choroid and the neurosensory retina, which allows for the immune privilege of the eye, as well as a selective transport between the neural retina and the choroid. Finally, the RPE cells phagocytose and digest the photoreceptor outer segments that shed and get renewed constantly during the visual cycle. This process will be explored further in the next section [5].

1.1.7 Phototransduction

The phototransduction process begins when light reaches the outer segments of the photoreceptors. When the photons are detected at the level of the opsins, they go through a conformational change, during which retinal undergoes isomerisation from 11-cis-retinal to all-trans-retinal. This leads to the hyperpolarisation of the membrane potential of the photoreceptor, which is the process by which the cell membrane potential becomes more negative than its resting state due to the closure of gated ion channels, reducing the influx of Ca^{2+} . This hyperpolarisation stimulates the release of a neurotransmitter called glutamate into the synapses and onto the bipolar cells, and the amount of glutamate released varies depending on the membrane potential of the photoreceptor cells. In light, photoreceptors hyperpolarise (more negative membrane potential) and release a lower amount of glutamate onto the bipolar cells. In contrast, in the dark, photoreceptors depolarise (more positive membrane potential) and release a higher amount of glutamate onto the bipolar cells. As a part of the phototransduction process, the outer segments of these photoreceptors shed and are phagocytosed by the RPE cells, which allows the all-trans-retinal to return to its 11-cis-retinal form [6].

Bipolar cells are retinal interneurons that respond to the glutamate released by the photoreceptors and transmit the signal onto the ganglion cells, allowing the visual signal to be transmitted further to the brain. There are two types of bipolar cells: ON and OFF. Rods can only interact with ON type bipolar cells, while cones can interact with both types. These two types of bipolar cells express different types of glutamate receptors

and thus respond oppositely to the glutamate released by the photoreceptors. In OFF bipolar cells, the glutamate release is excitatory, which means that in the dark, as there is more glutamate released, cation channels are open, and the membrane potential of the OFF bipolar cells depolarises (more positive membrane potential). In contrast, in the light, less glutamate is released which causes the channels to close, leading to hyperpolarisation of the membrane potential of the OFF bipolar cells (more negative membrane potential). ON bipolar cells behave in the opposite manner, as the glutamate release is inhibitory. This means that in the dark, the higher levels of glutamate lead to the closing of the cation channels, hyperpolarising the membrane potential of the ON bipolar cells (more negative membrane potential), while in the light, the lower levels of glutamate lead to the opening of these channels, depolarising the membrane potential of the ON bipolar cells (more positive membrane potential). The signal from the bipolar cells travels to the ganglion cells and finally to the optic nerve. Like the bipolar cells, there are two types of ganglion cells: Off-centre ganglion cells decrease their firing rate when more light is present, while ON-centre ganglion cells increase their firing rate [6].

1.2 The diseases affecting the retina

1.2.1 An introduction to the diseases

The visual system is the part of the central nervous system that detects and interprets visible light and converts it into a representation of the surrounding environment. Vision can be lost from several disorders including but not limited to age-related macular degeneration (affecting the retina), cataract (affecting the lens), diabetic retinopathy and glaucoma (affecting the optic nerve), in addition to several inherited retinal diseases (IRD). It is estimated that 34 million individuals within the European Union are affected by photoreceptor degenerative diseases, namely AMD and IRD [7].

One of the main causes for retinal degenerative diseases is irreversible loss of photoreceptors, which results in visual impairment, and can even cause blindness. More specifically, age-related macular degeneration is the leading cause of blindness

worldwide in individuals over the age of fifty, as well as the third overall leading cause of blindness [8]. Age-related macular disorder (AMD) is a late onset retinal disease and is caused by a combination of genetic and environmental factors [9], while IRD are neurodegenerative conditions that affect the retina. They are caused by genetic mutations of genes involved in retinal development, photoreceptor maintenance and phototransduction [10]. These include retinitis pigmentosa, Stargardt disease, Leber congenital amaurosis, as well as several more. Unlike AMD, which only affects the elderly, IRD can be diagnosed in early adulthood, and few can be diagnosed in childhood [10].

1.2.1.1 Age related macular degeneration

Age related macular degeneration is a degenerative disorder of the retina that affects photoreceptors as well as RPE cells and the Bruch's membrane. There are two types of AMD (Figure 1.5); dry AMD, which happens gradually over several years, and wet AMD, which happens rapidly over the course of a few weeks or months, and develops from the dry form [2]. Approximately 170 million people are affected by AMD around the world, making this disease the third leading cause of vision loss [11].

Dry AMD is caused by the formation and accumulation of lipid rich extracellular deposits, called drusen, in the macula. As this layer of drusen is deposited, it eventually blocks the Bruch's membrane, leading to localized inflammation, and disrupting the flow of oxygen and nutrients from the blood vessels to the cells, as well as the reverse flow of waste and carbon dioxide to the blood vessels [2], [12].

As this disease affects the macula primarily, the cone photoreceptors are the cells most affected, and this causes progressive loss of central vision over the course of several years. Once these cone photoreceptors die, the surrounding cells are not able to multiply into new photoreceptors to replace them because photoreceptors are post mitotic cells

that are no longer able to divide, therefore, the loss of these cells is permanent. This is what's known as late-stage dry AMD [2], [12].

Wet AMD is caused by the growth of abnormal blood vessels from the choroid underneath the macula into the subretinal space. Over time, these blood vessels rupture and cause scarring and the death of retinal cells, which also leads to irreversible loss of central vision. AMD also causes the death of the RPE cells and damage to the Bruch's membrane, and any form of therapy to restore vision needs to replace or regenerate all three layers [2], [12]–[15].

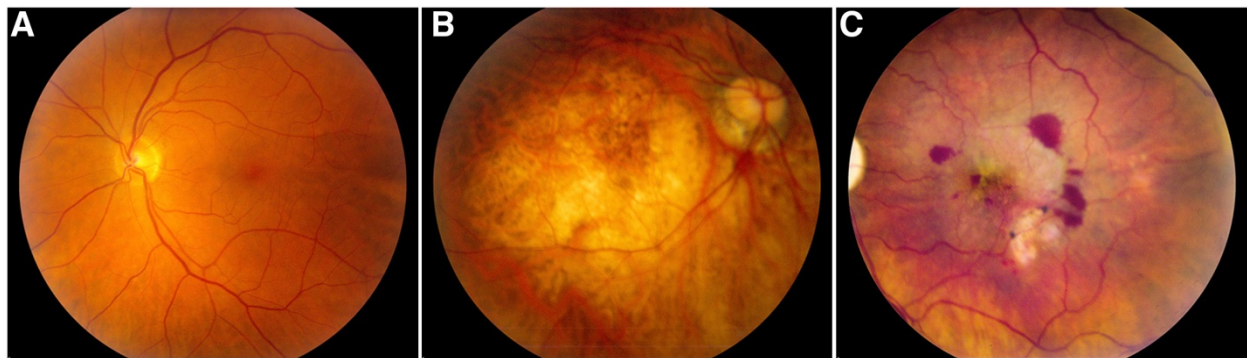


Figure 1.5: Fondus photographs of the eye. (A): Images of a healthy eye, with normal pigmentation and blood vessels. (B): Images of an eye with late-stage dry AMD, with visible depigmentation in the macula. (C): Images of an eye with wet AMD, with visible leaking blood vessels. Figure adapted with permission from [12].

1.2.1.2 Stargardt disease

Stargardt disease (STGD) is an inherited disorder of the retina that affects both photoreceptor and RPE cells. It is an autosomal recessive disorder due to mutations in the ABCA4 gene and can affect both adults and children. This mutation is the most common form of IRD and the most common single gene retinal degeneration. This

disorder causes progressive damage of the macula, leading to a loss in vision. In a normal, healthy eye, the ABCA4 protein is localised to the rods and cones' outer segments and has a function to clear the retinoids from the photoreceptors [16]. Depletion of ABCA4 causes cells to accumulate retinoid bi-products (lipofuscin fluorophores A2E and A2PE-H₂ in the RPE, and phosphatidylethanolamine (PE) and *N*-retinylidene-PE in the retina) which disrupts catalytic or retinoid binding function, leading to RPE cell dysfunction, their inability to maintain photoreceptor cell and ultimately to visual impairment [5], [17], [18].

1.2.2 Current treatments for retinal diseases

AMD and IRD are the leading causes of blindness worldwide and affect a high proportion of the population, and although loss of vision is not life threatening, it can severely affect a patient's quality of life. Once the retina is diseased, the photoreceptors die irreversibly, the retina suffers permanent remodeling of the remaining neurons and is unable to repair itself or regenerate [19].

Currently, treatment options for photoreceptor degenerative diseases are limited, while some therapies are in clinical trial. However, many of these therapies cannot be used for late-stage AMD, as these therapies target photoreceptors that are no longer present, and once these cells are lost, it is an irreversible process. Thus, a large percentage of the diseased individuals are not able to benefit from these treatments. There has been a lot of research conducted on developing possible treatments, some strategies have already been approved, some are in clinical trials whilst others are still being tested in the lab, however there is still no real cure to treat these diseases [20], [21].

1.2.2.1 VEGF inhibitors

The most successfully used treatment for wet AMD is anti-VEGF (vascular endothelial growth factor) injections into the vitreous cavity [8]. As wet AMD is characterized by the abnormal growth of blood vessels, these injections inhibit the action of VEGF in the eye

and stop the further growth and development of these vessels, slowing down the progress of the disease and delaying the death of photoreceptors. It is not a cure, but it is a commonly used and approved therapy that has improved the quality of life as well as the vision of millions of patients [7].

Currently, there are four anti-VEGF therapies approved by the FDA: brolucizumab (Beovu®), aflibercept (Eylea®), ranibizumab (Lucentis®), and pegaptanib (Macugen®). However, this form of treatment is only used for wet AMD, which only affects about 10-15% of all AMD patients [12], and usually at the end stages of macular degeneration. It cannot restore vision decline due to loss of photoreceptors or restore those lost cells. Also, this treatment requires regular injections, and up to 25% of patients do not respond to the treatment and continue to have a declining vision [7].

1.2.2.2 Gene therapies

Gene therapy is a medical field that treats diseases and prevents them by modification of the defective genetic material in cells to correct the underlying genetic condition. This is possible due to the development of an adeno-associated virus (AAV) for treatments [22]. AAVs are small, non-pathogenic viruses that are used as vectors for delivery of a normal copy of a gene into the nucleus of a host cell [23]. However, gene therapy is dependent on the health of the photoreceptor target cells, as it slows down the progression of the disease by targeting its genes.

The most successful gene therapy approach used for the retina is to treat Leber's congenital amaurosis (LCA) [24], and it was the first ocular gene therapy readily available in the market following approval by the FDA in late 2017 [7]. It works by targeting the RPE65 mutation that causes LCA (an inherited form of retinal degeneration) using AAV vectors, to introduce a healthy copy of the gene to the RPE cells through transfection. This therapy did initially achieve improvement in the visual function [25], however, there were reports of declining vision and continuing photoreceptor degeneration three years following the start of the trials [26]. Another challenge of using this vector is the maximum

genetic cargo size that the virus can carry, which is much smaller than the size of most of the genes that cause retinal degeneration [7].

Other gene therapy approaches are being studied, with the most prominent one being CRISPR/Cas technology, used to edit genes in patients with dominant and recessive forms of IRD [27]–[29]. CRISPR/Cas genome editing has been studied in animal models, however delivering the system directly into the target cells in the retina poses a challenge for researchers [28]. Furthermore, this therapy requires sufficient photoreceptor cells to be present in the retina, and therefore can only be used in early stages of the diseases. An additional challenge in using CRISPR/Cas gene editing technology is the off-target effects observed, as they can lead to unwanted modifications in genes that are similar to the targeted sequence, and could ultimately lead to harmful consequences [28], [29]. This genome editing approach also raises ethical concerns regarding its potential misuse, particularly when performed on human embryos to create ‘designer babies’ [29].

Optogenetic tools present a great potential alternative, as this strategy aims to confer light-sensitivity to the remaining non-light sensitive interneurons in the retina (including bipolar cells and retinal ganglion cells) by delivering genetically encoded light-activated proteins into those cells, altering their function to become photoreceptor-like [30]. This therapeutic approach is different to the other forms of gene therapy because it does not require any photoreceptor cells to be present in the retina, meaning it can be used to treat late stages of macular degeneration. However, the optogenetic tools currently available still do not present the necessary sensitivity to provide a level of vision comparable to that photoreceptor cells provide, and a large amount of light is needed to induce a response, which can be damaging to the eye [31], [32].

Neuroprotective therapies are also used to treat some neurodegenerative conditions in the eye, including glaucoma, AMD and diabetic retinopathy [33]. The therapy works by protecting the nerve cell from damage, chronic or acute, allowing the cell to restore normal physiology. These therapies can inhibit necrosis and apoptosis through different

pathways, preventing loss of photoreceptors [8]. This form of treatment is used to treat AMD, and aims to stop the progression of diseases, and maintain the patient's vision. However, it cannot restore any lost visual functions. AMD is the only retinal disease with a neuroprotective agent approved by the FDA [24]. This oral supplement (AREDS), containing vitamin C, vitamin E, zinc oxide, and other chemicals, has been reported to slow down the advancement of wet AMD by 25% [34].

1.2.2.3 Prostheses

Implantable electronic retinal prostheses have been widely investigated to restore vision in people with blindness and have evolved significantly since they were first introduced [35]. Currently, several devices have been approved for use in Europe or the United States. These prostheses can be divided into three types, epiretinal, subretinal and suprachoroidal, all containing two dimensional arrays of electrodes.

Epiretinal implants are placed on the surface of the retina and stimulate the retinal ganglion cells directly. These devices bypass the degenerating photoreceptor layer, making them a good choice for late stages of retinal degeneration. However, unlike natural vision, the patient is required to move their head to shift their vision and locate objects. The Argus II retinal prosthesis system (USA) was the first epiretinal device to receive the CE marking in 2011 and FEA approval in 2013. This device is also the most widely used across the world, with over 250 individuals having received the device [35]. The Argus II phase 2 clinical trial consisted of 30 patients and evaluated the safety of the implanted devices and their effect on vision and on task performance. This trial resulted in improvement in the patients' vision and performance, with further follow-ups conducted at 5 years and 10 years post implantation. Some adverse events were observed throughout the trial, however they were all treatable with standard protocols [35].

Subretinal implants are placed in the subretinal space between the photoreceptor layer and RPE layer. This implant stimulates all the remaining cells of the neural retina, and

rely on the normal processing of the eye, and normal eye movement to shift the patient's gaze. However, these implants require sufficient incident light, and require an external device to amplify the signal [35]. The Alpha IMS is the first of these subretinal implants to receive the CE marking in 2013, following clinical trials from 2010 to 2014 aimed to evaluate the safety and efficacy of the implanted device. This trial consisted of 36 individuals, with 86% reporting significant improvement in light perception with the device [35].

Suprachoroidal implants are placed between the sclera and the choroid, requiring less invasive surgery, and making it more easily accessible for repair when needed. However, the placement of these implants at a larger distance from the neural retina requires a larger stimulation power and tend to yield low spatial resolution [35]. Some pilot studies with few patients were conducted with suprachoroidal implants, with results suggesting some improvement in vision. However, results to date are not enough to draw clear conclusions about the long-term effect of these implants [35].

All these prostheses show promising results, with clinical trials demonstrating some success with all three of these devices, and patients regaining some of their visual function. However, they have the same limitation: low spatial and temporal resolution. Therefore, the visual improvement demonstrated in the trials is still inferior to natural vision and can only be improved further by increasing the number and size of the implanted electrodes, which would cause more damage to the retinal tissue through heat generation. This technology still requires optimization but has shown progress over the last 25 years [35].

1.2.3 Conclusion

Several different treatments to treat macular degeneration diseases have been studied and many are in clinical trials, to halt the progression of the diseases. However, there are limited clinical treatments for restoring normal vision in patients affected by macular

disorders, and those that do provide low resolution vision. Therefore, the most promising strategies to address these disorders and potentially restore the damaged vision are cell replacement therapies. This is possible due to the non-synaptic layer in the retina, made up of the RPE cells and photoreceptor outer segments, that can allow for photoreceptor transplantations [36]. This is also facilitated by the immune privilege property of the eye, which means it has limited immune response towards the transplanted cells.

1.3 Strategies for photoreceptor delivery

Various cell replacement therapies for retinal degeneration have been investigated over the past two decades. These strategies, summarised in Figure 1.6, differ in terms of the cell source and delivery methods. In most rodent model studies, the transplanted cells or retina sheets are obtained from mouse primary cells or derived from mouse or human pluripotent stem cells. Retinal sheets are transplanted directly into the subretinal space, while the cells in suspension require an enrichment step before they can be transplanted, either through FACS or MACS. These strategies will be described in more detail in this section.

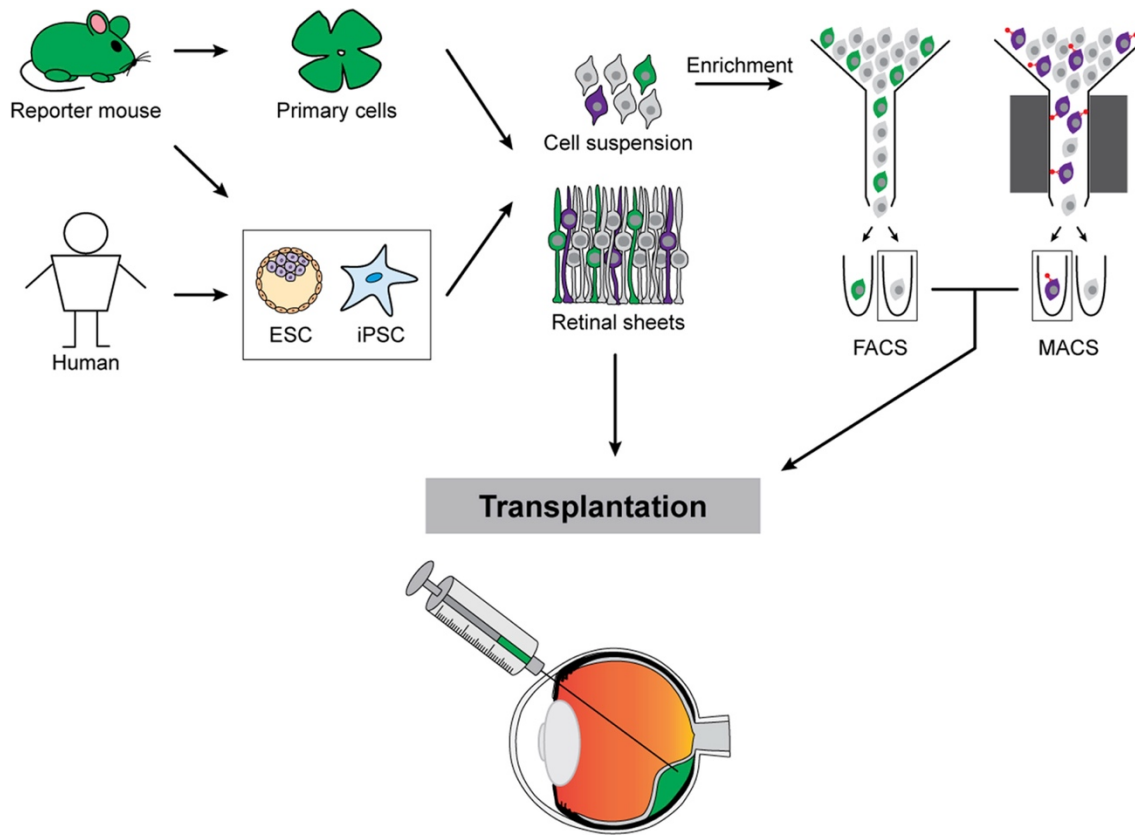


Figure 1.6: Overview of photoreceptor transplantation in rodent models. The transplanted cells or retina sheets are obtained from mouse primary cells or derived from mouse or human pluripotent stem cells. Retinal sheets are transplanted directly into the subretinal space, while the cells in suspension require an enrichment step before they can be transplanted, either through FACS or MACS. Figure adapted from [7] under the creative commons attribution-noncommercial-no derivatives licence.

1.3.1 Cell sources for photoreceptor transplantation

1.3.1.1 Primary cells isolated from mouse retinas

The first publications of photoreceptor transplantation into rodent models were in the late 80s, with del Corro *et al.* (1988) reporting survival and integration of fluorescently

labelled donor cells, derived from P0-2 postnatal rats, into the damaged retinas of rats for up to 100 days [37]. These early results inspired many studies to follow, including transplantations of retinal progenitor cells [38]–[40], and neural-stem precursors [41]. Whilst these transplanted cells survived in the host retina, they failed to differentiate into mature photoreceptors [42]–[44].

In 2006, MacLaren *et al.* identified the optimal age of donor cells for photoreceptor transplantations. They found that photoreceptor precursors, or immature postmitotic photoreceptors, yielded higher survival and integration rates once transplanted into the retina [45], [46]. This optimal stage for precursor isolation and transplantation was reported as post-natal day 4 to 8 (P4-8) for rod photoreceptors, as these cells were committed, but not yet fully differentiated [45], [47], [48].

To assess the success of transplantations, these photoreceptor precursor cells were isolated and purified from transgenic mice, modified to express a green fluorescent protein (GFP). Akimoto *et al.* (2006) developed Nrl.GFP^{+/+} mice, where the promoter for the neural retina leucine zipper (Nrl) transcription factor activates the expression of GFP in post-mitotic rod photoreceptors [49]. Further studies developed a Crx.GFP^{+/+} (cone-rod homeobox) transgenic mouse line to isolate both rod and cone photoreceptors expressing GFP [50]–[52]. In both cases, the transplanted photoreceptor cells expressed a protein absent in the host cells, allowing for better evaluation of successful photoreceptor integration within the host outer nuclear layer [44].

This primary photoreceptor isolation method could not be translated to humans, as the optimal developmental stage for photoreceptors would fall into the second trimester of pregnancy [7], posing significant concerns, ethically and logistically. Therefore, alternative cell sources were studied. Embryonic stem cells (ECS) and induced pluripotent stem cells (iPSC) were a more promising source of photoreceptor cells.

1.3.1.2 Pluripotent stem cells

Several research groups focused on developing 2D photoreceptor differentiation protocols from mouse and human ESC/iPSCs, with various outcomes and efficiencies [39], [53]–[56]. Lamba *et al.* (2009) attempted the first transplantation of EGFP expressing photoreceptors, derived from hESCs. These cells showed evidence of integration into the host outer nuclear layer and displayed the morphology of mature photoreceptor cells [57]. West *et al.* (2012) transplanted mESCs-derived photoreceptor precursor cells into mice models, however, they observed no integrated cells in the host retina [55]. Further studies reported transplantations of iPSC-derived photoreceptors with varying degrees of success [58], [59].

In 2011 and 2012, the Sasai group published ground-breaking methods to differentiate mESCs and hESCs in a three-dimensional (3D) culture system [60]–[62]. Their protocols demonstrated the capability of stem cells to self-organise and differentiate by forming retinal organoids, that can develop into bilayer optic cups, resulting in an organised retinal tissue, as seen in Figure 1.7. Since then, several research groups have adapted this 3D retinal organoid technology to generate transplantable photoreceptors [51], [63]–[65].

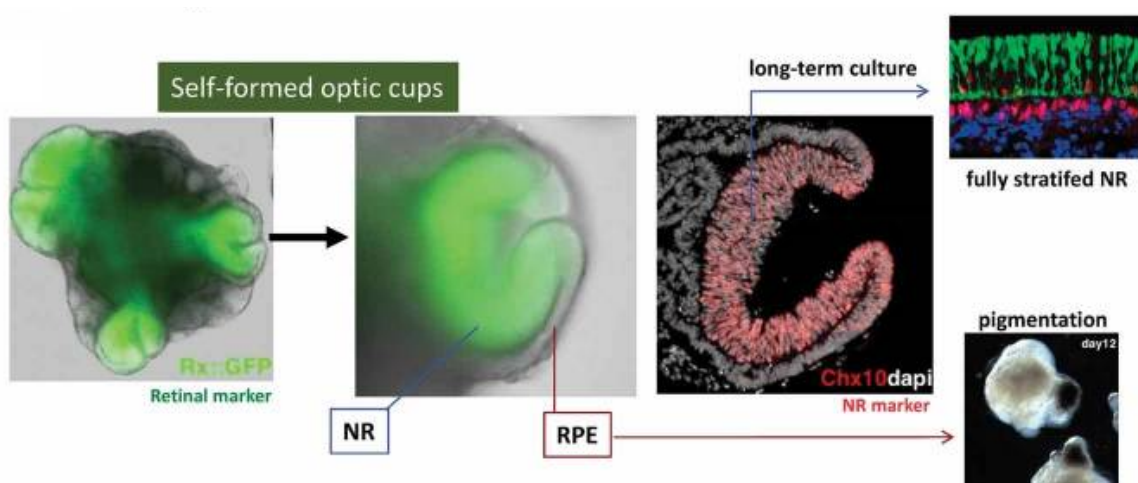


Figure 1.7: ESC derived optic cups. Each cup has an inner stratified neural retina (NR) and an outer RPE wall. Green Rx::GFP for retinal marking. Figure adapted with permission from [3].

Gonzalez-Cordero *et al.* (2013) adapted this method to generate GFP positive post-mitotic rod precursor cells from mESC cultures. Once transplanted, these cells were found within the host ONL and compared to primary photoreceptor transplantation, did show similar results [63]. Furthermore, Decembrini *et al.* (2014) isolated and transplanted GFP positive cells from mouse retinal organoids and observed cellular integration and maturation in the host retina [51]. Similar results were observed by Lakowski *et al.* (2015) and Kruczek *et al.* (2017) [66], [67]. Gonzalez-Cordero *et al.* (2017) also optimised the protocol for human retinal organoid-derived photoreceptors and reported cell maturation following transplantation [68]. One of the challenges with producing human ECS-derived retinal organoids is the extended culture times required, ranging from 60 to 80 days for cone, and up to 200 days for rods [44].

1.3.2 Photoreceptor transplantation approaches

The second aspect of photoreceptor transplantations studied is the approach used to deliver the cells into the host retina. The eye, being an immune privileged organ, increases the chances of the transplantations surviving in the subretinal space [69]. Two transplantation approaches are used and will be explained further below.

1.3.2.1 Single cell transplantation

Single cell transplantations have several advantages over sheet transplantation strategies. These include transplantations of a defined number of cells, minimal surgical instrumentation and trauma, and better contact between the transplanted cells and the host cells [7].

Using single cells in suspension also require cell enrichment prior to transplantation. Two strategies are commonly used for photoreceptor cells: fluorescence activated cell sorting (FACS), and magnetic activated cell sorting (MACS). The development of the transgenic Nrl.GFP^{+/+} mouse line made it possible to easily isolate and purify rod post-mitotic

photoreceptors using FACS [49]. However, FACS causes significant shear stress to the cells and is a relatively slow process [70]. MACS is a gentler approach and uses the CD73 cell surface marker to sort photoreceptors. It has been shown to produce enriched cell populations with high viability from both primary mouse retinas and mouse ESC derived retinal organoids [71], [72].

Early single cell transplantation results were very promising, showing that transplanted cells could integrate into to host ONL and mature [45], [47], [48], [63]. Additionally, functional or behaviour tests suggested rescue of rod-mediated vision following transplantation [45], [47], [48], with Pearson *et al.* (2012) focusing on characterising the level of rescue. However, all these results needed to be reassessed due to findings from Pearson *et al.* and Santos-Ferreira *et al.*, both published in 2016 [65], [73]. Up until 2016, it was believed that GFP positive cells observed in the ONL of the host retina were the transplanted photoreceptor cells, and that their integration resulted in the visual improvement detected. However, these findings established that the majority of the GFP positive cells observed were host photoreceptors. These results indicate that there was cytoplasmic material transfer of RNA and/or protein between the transplanted cells and the host cells [65], [73].

Single cell transplantation methods require bolus injections of the suspension into the host retina, which comes with some disadvantages. These include efflux or leakage of the transplanted cells at the injection site [40], [74], [75], high cell death [40], [75], [76], migration of the cells from the transplantation site [40], [75], and poor integration and limited long-term survival [77], due to inhibitory ECM proteins [48], [75].

1.3.2.2 Sheet transplantation

Retinal sheets transplantations have been studied as an alternative to single cell suspensions because they conserve the organised structure of the retinal cells and can be more effective when transplanted in advanced stages of retinal degeneration [64].

These sheets can be manually dissected from a retinal organoid [64] or a fetal retina [78], [79] and transplanted as a whole unit.

The first paper describing this approach was published in 1898. Silverman and Hughes (1989) developed a method to isolate photoreceptor sheets from an 8-day old rat and from an adult rat, using gelatin embedding and vibratome sectioning [78]. These sheets were then transplanted into rats with damaged retinas, however, did not maintain outer segments *in vivo*. More precise methods for isolating the photoreceptor layer were later developed using an excimer laser [79].

This transplantation approach offers advantages including long term graft survival, with Ghosh *et al.* (2007) studying the long-term transplantation of a fetal retinal sheet in pigs [80]. They reported normal laminated morphology within the graft 6 months post transplantation, however no connections were formed between the host and transplanted cells [80]. This represents one of the disadvantages to using this transplantation strategy, as the transplanted retinal sheets not only contain a photoreceptor layer, but several layers of retinal cells. Once transplanted into the host retina, this leads to the formation of a 'double retina', with multiple layers of the same cells overlapping [7], [64].

Additionally, Assawachananont *et al.* (2014) demonstrated that mouse ESC or iPSC derived retinal sheets, with a structured ONL, could be transplanted into mouse models with advanced retinal degeneration (rd1) [64]. These sheets survived in the host retina and could form synaptic connections with the host cells. This demonstrates another advantage of using retinal sheets, as there is limited evidence of cell suspensions restoring lost vision in rd1 mouse models [48]. However, these transplanted retinal sheets tend to develop rosettes, especially post-transplantation into the subretinal space of rd1 mice. This hinders the formation of synaptic connections between the transplanted photoreceptor cells and the host neural retina, as well as interactions

between these photoreceptors and the host RPE cells, necessary for the visual cycle [64].

1.4 Transplantations with biomaterials

Photoreceptor transplantations have been studied extensively in the literature; however, many challenges still limit their integration into the host retina, and their interactions with the rest of the neural retina, necessary for restoring vision. Therefore, biomaterial support has been explored to improve these replacement therapies. These scaffolds can facilitate the delivery and transplantation of the donor photoreceptor cells into the retina in a more controlled and structured way, and some materials can also release factors to increase cell survival.

1.4.1 Non patterned scaffolds

In early studies, retinal sheets were transplanted into the host retina using biomaterials. The sheets were embedded into gelatin and using vibratome sectioning, the unwanted retinal layers were removed until the photoreceptor layer was isolated and maintained in the gelatin [78]. This allowed the layer to be more easily manipulated during the transplantation without damaging the photoreceptor cells. The gelatin layer provided protection to the cells and dissolved in the body (due to body temperature), without causing any cytotoxicity. However, the organisation of these sheets was not maintained *in vivo*, with approximately half the transplanted photoreceptor layers forming rosettes. Nevertheless, the transplanted photoreceptor cells were found to survive, integrate, and produce opsin, signifying their potential to transduce light [78].

Further studies continued to investigate transplanting retinal sheets with biomaterial support. Pritchard *et al.* (2010) isolated photoreceptor layers *in vitro* and cultured them on a bi-layer biomaterial membrane to create a transplantable construct. Their material consisted of a 30 μm thick poly(glycerol-co-sebacic acid) (PGS) disk, onto which a layer

of poly(epsilon-caprolactone) (PCL), blended with 10 wt% laminin, was electrospun (fibre diameters of approximately 100 nm), to promote the attachment of the cell layer to the biomaterials. The retinal layers attached to the membranes to after 5 to 7 days of culture and were successfully transplanted into the subretinal space of porcine animal models. The transplanted constructs did integrate into the retinas with no signs of inflammation, and three months post surgeries, the scaffold was fully degraded, leaving behind the integrated photoreceptor layer. However, the same behaviour described in Silverman 1989 was observed, with the transplanted photoreceptor layer forming rosettes, even prior to transplantation [81].

Later biomaterial studies favoured using retinal progenitor cells (RPCs), as several publications demonstrated the ability of these cells to migrate into the host retina and integrate with the host cells and forming connections [82], [83]. However, as described previously, simple bolus injections of these cells did not yield a large percentage of successfully integrated cells. Therefore, the need for biomaterial support grew, with several biomaterials being researched for this purpose. Some groups favoured using synthetic polymer scaffolds, while others preferred natural polymers.

One of the leading research groups focusing on synthetic polymer scaffolds for RPC transplantation is the Young group. This group has published several papers describing different approaches and materials used to culture and transplant RPCs into animal models. In their 2008 paper, the authors cultured mouse RPCs on nanowire poly(epsilon-caprolactone) (PCL) scaffolds, coated with laminin, and transplanted the scaffold into mouse model retinas. These scaffolds resulted in proliferation of the seeded cells, and increased survival of the cells post transplantation [74].

Another material tested extensively by the Young group was poly(lactic-co-glycolic acid) (PLGA), as it is an FDA approved biocompatible and biodegradable copolymer [84]. Their initial experiments consisted of culturing RPCs on PLGA scaffolds for up to 14 days and transplanting the cell/material constructs into the subretinal space of mouse and rat

models to study the survival, integration, and migration of these cells in the host retina [85], [86]. The transplanted RPCs demonstrated a 10-fold increase in the number of surviving cells 4 weeks post transplantation compared to bolus injections, and a 16-fold increase in the number of cells delivered [86], indicating the advantages of using PLGA scaffolds.

Additionally, the group studied the effect of photoreceptor degeneration on the production of inhibitory ECM matrix and cell adhesion molecules that act as a barrier to the migration of the transplanted cells into the damaged retina and preventing its regeneration [75], [87]. Therefore, the authors used PLGA not only as a scaffold for RPC culture and delivery but also as a delivery vehicle for matrix metalloproteinase 2 (MMP2), for the purpose of degrading the inhibitory ECM and cell adhesion molecules. They reported successful results, both in terms of MMP2 degradation (without causing damage to the retina), as well as increased migration of RPCs into the host retina. However, the transplanted RPCs did not all differentiate into rhodopsin-positive cells (rod photoreceptors), and they migrated to the inner nuclear layer instead of remaining in the outer nuclear layer, which could negatively affect the host cells and lead to the loss of bipolar cells [87]. This behaviour could be attributed to the use of RPCs instead of more mature photoreceptor cells for the transplantation studies.

The Young group also explored using a natural polymer, hyaluronic acid, as it is one of the main components of the ECM, and plays a vital role in cell proliferation, migration, and wound healing [88]. In their 2013 paper, the authors encapsulated RPCs into hyaluronic acid-based hydrogels and transplanted the scaffolds into mouse retinas. As these hydrogels were not crosslinked, they were a viscous solution that was easily injectable into the subretinal space without disrupting the host retina. Three weeks post transplantation, the hydrogels were degraded, and the cells were able to integrate, differentiate, and expressed recoverin, a marker for mature photoreceptors. However, similarly to Yao *et al.*, the cells did not integrate into the outer nuclear layer and did not express rhodopsin [89].

Another group that has been focusing on hyaluronic acid scaffolds for photoreceptor transplantations is the Shoichet group, with early papers published over a decade ago. Their initial experiments utilised both hyaluronan (HA) and methylcellulose (MC) to transplant retinal stem-progenitor cells (RSPC) into the subretinal space [40], [42]. Their blended materials (HAMC) formed an injectable viscous solution that degraded post transplantation, with the HA retaining 3% of its initial mass after 7 days, and the MC retaining 20% [40]. Their 4-week post transplantation results indicated that the cells injected in the subretinal space were more evenly distributed and formed less aggregates, compared to cells injected in a saline solution [40]. Their 2015 follow-up paper, Ballios *et al.* demonstrated improvement in the survival and integration of transplanted retinal stem cell-derived rods using the same hyaluronan-based hydrogel when compared to simple cell injections in a saline solution [42]. Additional injections of DL-alpha-aminoadipic acid (AAA) to disrupt the outer limiting membrane layer facilitated cellular migration into the outer nuclear layer [43]. They attributed these results to the interactions of a cell adhesion receptor (CD44) with the hyaluronic acid [42].

Mitrousis *et al.*'s 2016 paper also established that hyaluronic acid-based hydrogels significantly increased photoreceptor survival and maturation *in vitro* compared to cultures on tissue culture plastic, with the cells exhibiting upregulation of outer segment markers [90]. Hyaluronic acid was modified with a furan so that the HA-furan could be crosslinked with poly(ethylene glycol) to form HAPEG hydrogels. Varying concentrations of HA-furan with several furan degrees of functionalisation were tested to determine the effect of increasing the hydrogel stiffness on photoreceptor cell survival. As seen in Figure 1.8 (A) and (B), increasing the hydrogel stiffness did not affect the viability of the photoreceptors. Additionally, the HAPEG hydrogels were compared to several conditions, including methylcellulose crosslinked with PEG (MCPEG) with and without 1% HA added to the gel mixture, and standard cell culture conditions with and without 1% HAMA added to the medium. The results, shown in Figure 1.8 (C) and (D), show that the addition of HA, either crosslinked in the hydrogel, or non-crosslinked in the MCPEG

and standard culture medium, increases the viability of the photoreceptors cultured on the hydrogels.

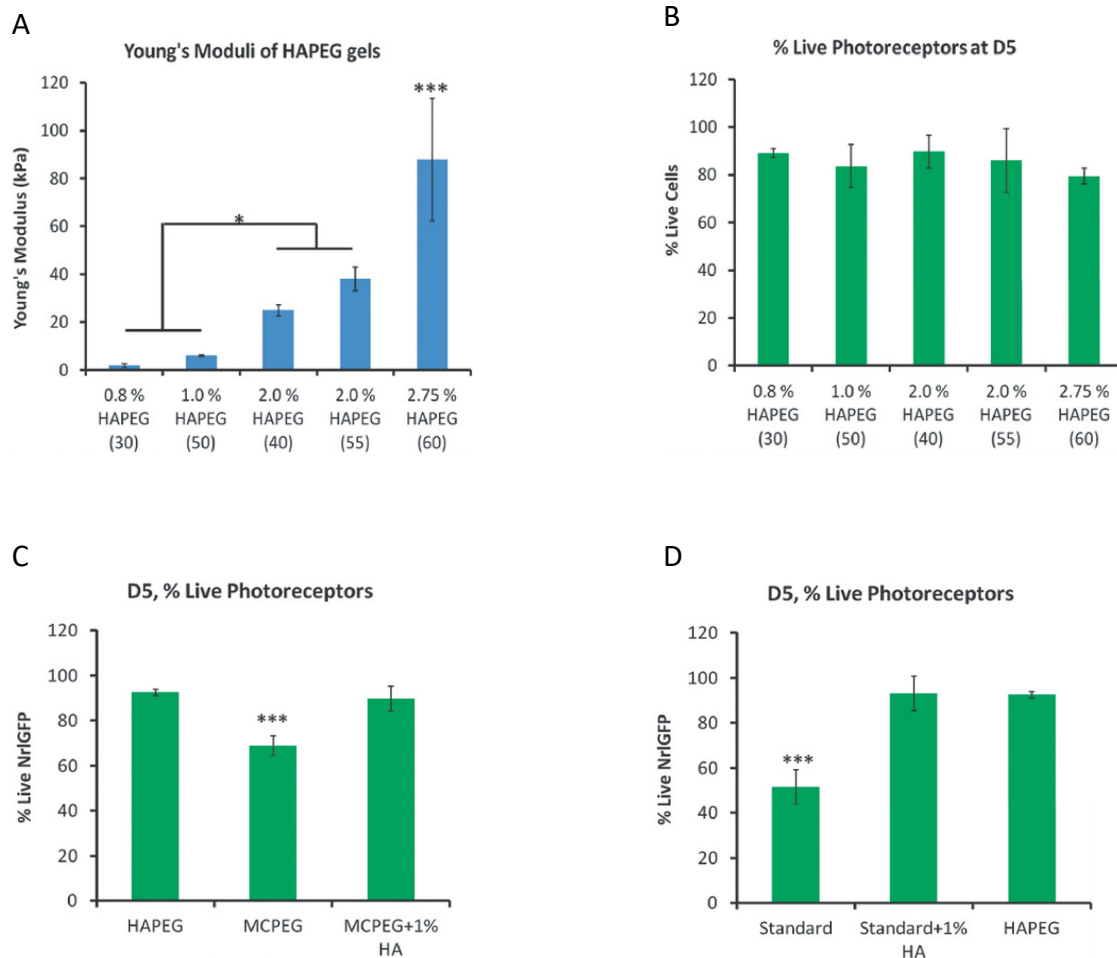


Figure 1.8: Testing the effect of hydrogel stiffness and HA presence on cell viability. (A): Young's moduli of HAPEG hydrogels with varying HA concentration and furan substitution (in parenthesis). (B): Photoreceptor viability at day 5 on the different HAPEG hydrogel conditions from (A). (C): Photoreceptor viability at day 5 on HAPEG gels, MCPEG gels, and MCPEG gels with 1% HA added to the gel mixture. (D): Photoreceptor viability at day 5 on HAPEG gels, standard cell culture conditions, and standard conditions with 1% HA added to the medium. Figure adapted with permission from [90].

Further hyaluronic acid-based hydrogels were explored, with more tuneable crosslinking methods and controlled mechanical properties [91]. The hyaluronic acid hydrogels were modified with either a norbornene or a methyltetrazine to allow for an inverse electron-demand Diels-Alder crosslinking reaction between the norbornene and the methyltetrazine. The authors reported that by optimising the hydrogel composition, it was possible to produce non swelling hydrogels (<1%), that were biocompatible and biodegradable. Additionally, primary photoreceptors that were encapsulated in the hydrogels had similar viability to those cultured on glass surfaces [91].

The Shoichet group continued to pursue HAMC hydrogels for both photoreceptor and retinal pigment epithelial cell transplantation. Primary photoreceptors were isolated from Nrl.GFP positive mice and co-transplanted with RPE cells, differentiated from human embryonic stem cells, in HAMC hydrogels. The scaffolds were injected into the subretinal space of mice. The mice were previously injected with sodium iodate to degenerate both the host photoreceptor and RPE layers resulting in blind mice with similar retinas to those in advanced stages of retinal degeneration. The experiments confirmed that the scaffolds containing both cell types displayed increased viability of both photoreceptors and RPE cells, compared to scaffolds injected with either one of the two cell types. Additionally, there was evidence of visual recovery in the co-transplanted scaffolds using behavioural tests [92].

1.4.2 Patterned scaffolds

The most significant challenge for transplanting photoreceptors into the host retina is achieving cell realignment after the cells have been sorted. During the sorting process, either with fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS), the photoreceptors lose their axons and their outer segments, leaving them with a spherical geometry. Once seeded on a surface or in scaffold, the cells are not able to regrow their processes in a controlled manner. Therefore, researchers have developed more sophisticated patterned scaffolds for the aim of guiding the growth of cellular

processes in a specific direction, with the intention of polarising the cells prior to transplantation and increasing the integration of these cells in the host retina.

The Young group have explored the effect of scaffold geometry on photoreceptor survival [93], [94]. A microporous poly(glycerol-sebacate) PGS scaffold was fabricated using soft lithography, with 50 μm holes spaced 175 μm apart, as seen in Figure 1.9 (A) and (B). Interestingly, this scaffold's properties allowed it to be scrolled and transplanted with the seeded cells, as seen in Figure 1.9 (C) and (D) [94].

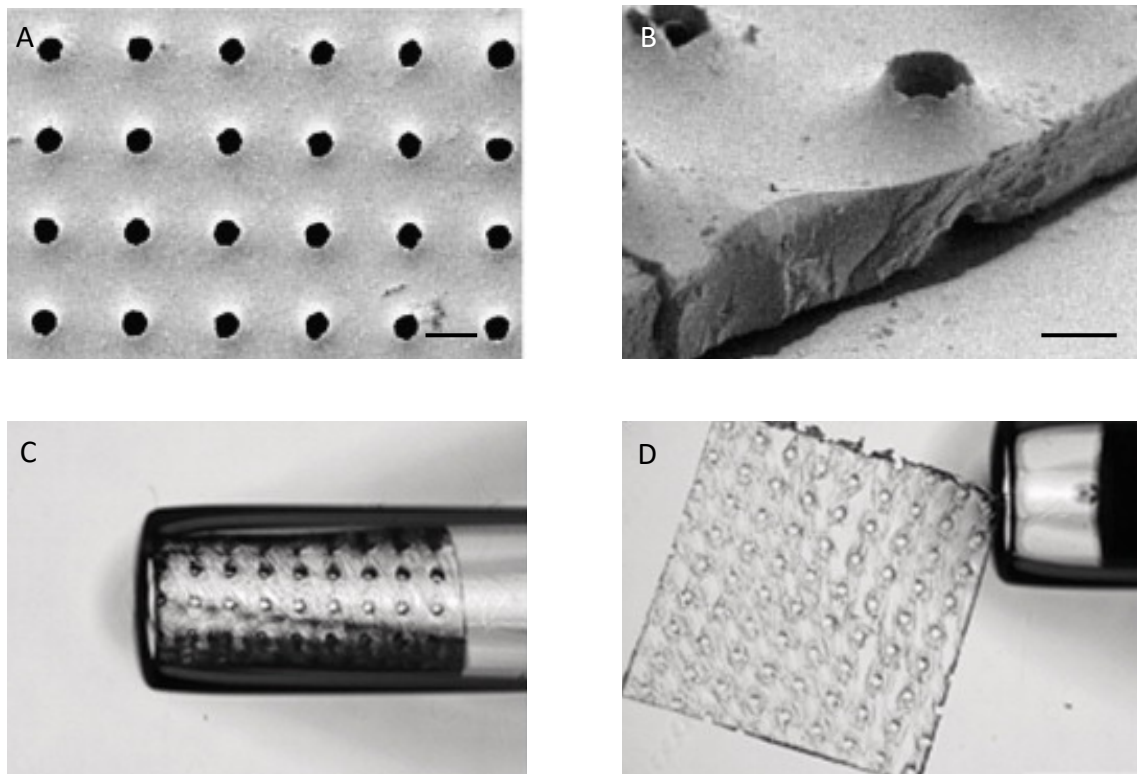


Figure 1.9: Images of the scrollable patterned PGS scaffold. The PGS scaffold was fabricated using soft lithography, with 50 μm holes spaced 175 μm apart. Scanning electron microscopy images of the PGS scaffold, viewed from the top (A) and the side (B). Scrolled (C) and un-scrolled (D) PGS scaffold in a glass needle with an inner diameter of 1 mm. Scale bars: (A): 100 μm , (B): 50 μm . Figure adapted with permission from [94].

The micropatterned PGS scaffolds, pre-coated with laminin, were seeded with RPCs from a GFP positive cell line for 7 days. Once completed, the cells had infiltrated the porous scaffold, maintained a high viability, and showed signs of partial differentiation. However, the cells lacked mature retinal markers such as recoverin and rhodopsin [93]. These scaffolds were also transplanted into the subretinal space of mice. After 30 days, no PGS was located in the subretinal space, and the transplanted cells expressed photoreceptor markers *in vivo*. However, the also expressed markers of bipolar cells and further studies to direct differentiation towards a photoreceptor fate should therefore be conducted [94].

Steedman *et al.* (2010) studied a PCL scaffold with superficial topographical cues running through the thickness of the scaffold [95]. RPCs were cultured with these scaffolds and resulted in higher cellular attachment compared to non-patterned PCL and tissue culture polystyrene. Furthermore, mRNA analysis showed increased expression of photoreceptor markers on both types of PCL scaffolds compared to tissue culture polystyrene. Despite their positive indications on using PCL as a material for RPC transplantations, this material is not an ideal option. PCL is a slow degrading material, with degradation rates measured in years instead of months, slowing down tissue regeneration in the transplanted area [96]. Furthermore, due to its hydrophobicity, it has limited ability to support cell growth and differentiation when compared to other biodegradable polymers used for RPC transplantations such as PLGA [84].

Yao *et al.* (2015) also studied the effect of patterned PCL scaffolds on RPCs [97]. As seen in Figure 1.10, they designed a scaffold with grooves and ridges and another with posts, both fabricated with photolithography and solvent casting, and compared both to smooth non-patterned PCL. RPCs cultured on all three scaffolds exhibited higher potential to differentiate towards a photoreceptor fate compared to glass controls. Furthermore, the grooves-ridges scaffold seemed to elongate and align the cells in the corresponding direction and showed the most promising potential for transplantations, as well as demonstrated the possibility of directing the growth of processes in a specific

direction. However, similarly to the Steedman *et al.* (2010) paper discussed above, PCL is not an optimal choice of biomaterial for RPC transplantations.

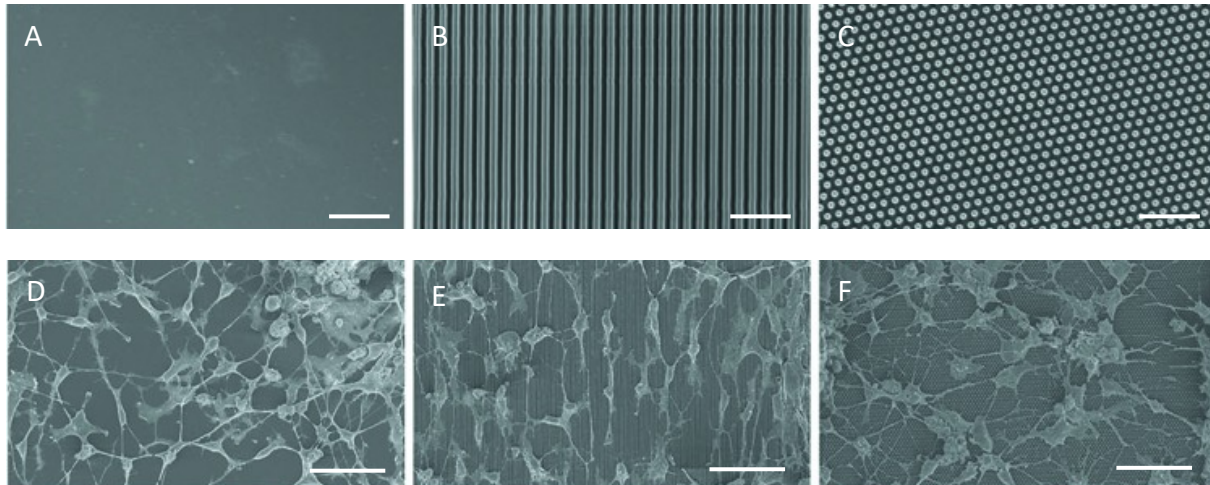


Figure 1.10: Images of the different PCL scaffold surfaces and cellular morphology. Scanning electron microscopy images of the PCL scaffolds, for smooth (A), ridge-groove (B), and post (C) PCL. Morphology of RPCs 7 days after seeding on scaffolds with smooth (A), ridge-groove (B), and post (C) PCL. The cells on the ridge-groove scaffold seemed to elongate and align the cells in the corresponding direction and showed the most promising potential for transplantations. Scale bars: (A): 200 μm , (B) and (C): 20 μm , (D), (E) and (F): 100 μm . Figure adapted with permission from [97].

Jung *et al.* (2018) have been successful in seeding human pluripotent stem cell derived photoreceptors in a patterned biomaterial scaffold to guide their polarisation in an apical-basal direction [98]. Using microfabrication techniques, a three-dimensional scaffold was developed from both polydimethylsiloxane (PDMS) and poly(glycerol-sebacate) (PGS), with the latter being a biodegradable elastomer used in other publications described above. The fabrication process is described in Figure 1.11 and uses photolithography and soft moulding to develop the patterned scaffold. Briefly, the silicon master mould was fabricated using reactive ion etching (isotropic and anisotropic) to create the

channels. The secondary mould was created using a mixture of hard and soft PDMS. Finally, the scaffold was fabricated by pressing the secondary mould into the biomaterial layer, curing the scaffold, and delaminating it. Scanning electron microscopy images of these moulds can be seen in Figure 1.11.

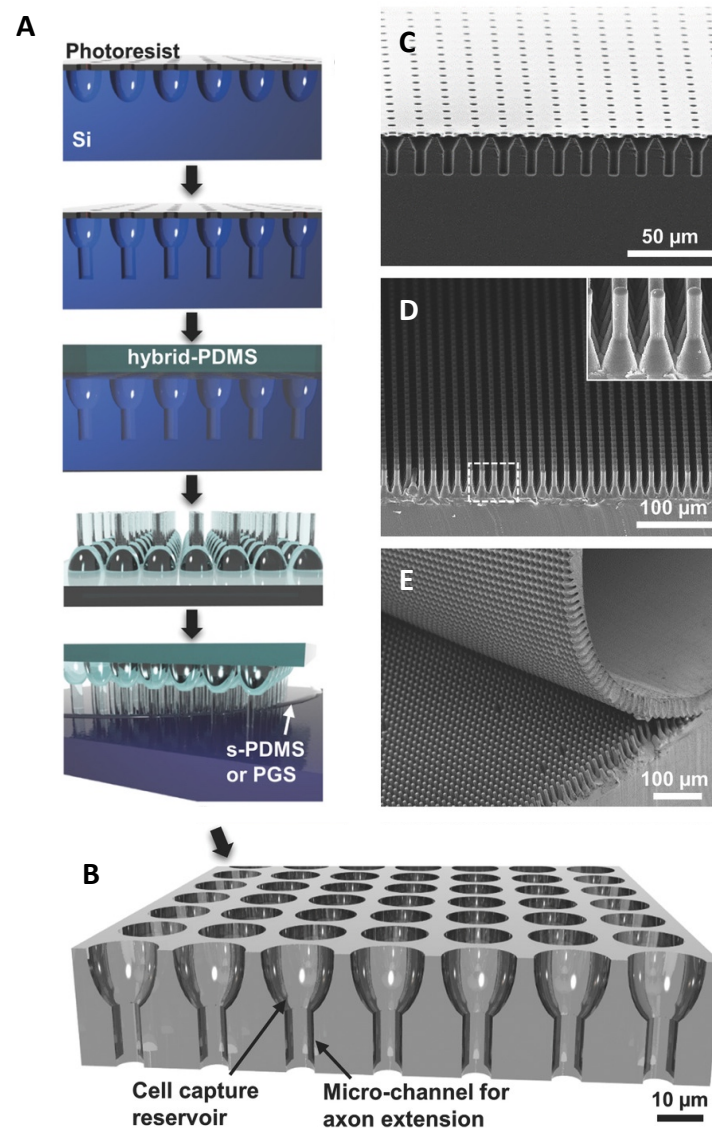


Figure 1.11: Fabrication of a patterned scaffold with 'wine glass' shaped channels. (A) Schematic of the microfabrication and soft moulding processes used in Jung 2018 to fabricate the micro-patterned scaffold design seen in (B). (C-E): Scanning electron microscopy images of the silicon master mould (C), the PDMS secondary mould (D), and the final patterned scaffold (E). Figure adapted with permission from [98].

The scaffold had a defined pattern of channels, shown in Figure 1.12, designed to induce photoreceptors polarisation once seeded. The individual channels had a ‘wine glass’ geometry, with a larger reservoir in the top half of the scaffold, and narrower channels in the bottom half. Their hypothesis was that seeding the photoreceptor cell bodies in the reservoir would induce the photoreceptors to grow their processes in the narrower channels [98].

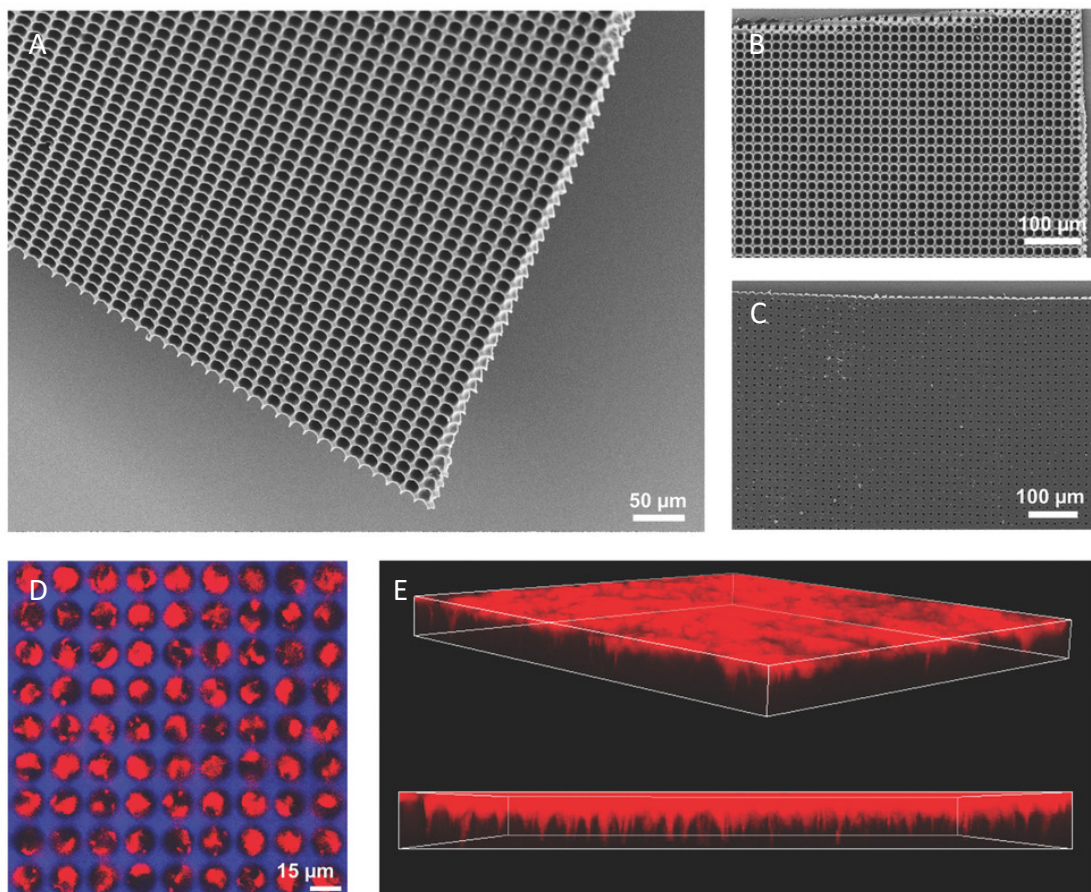


Figure 1.12: Images of a patterned PGS scaffold with cultured photoreceptors. Scanning electron microscopy images of the PGS scaffold taken at a tilted view (A), top view (B), and bottom view (C). (D): confocal image of the scaffolds seeded with photoreceptors expressing tdTomato after 9 days, viewed from the top. (E): confocal images of the scaffolds one month post seeding. Figure adapted with permission from [98].

To test the efficacy of this design, human pluripotent stem cell derived photoreceptors (hPSCs) were seeded in the scaffolds. The cells were collected and dissociated from retinal organoids that were 80 days old. The scaffolds were autoclaved, plasma treated and coated with laminin before the cells were seeded. The photoreceptors survived on the scaffold for at least three months *in vitro*, and continued to mature, with the cells continuing to express rod-specific markers. Additionally, the cells showed extension of their processes into the narrower channels and expression of presynaptic markers, as seen in Figure 1.12 [98].

The cell culture results demonstrated successful polarisation of photoreceptor cells in the micro-patterned scaffold. However, this study did not investigate the transplantation of this scaffold *in vivo*, to investigate whether these photoreceptors are able to form synapses with the host neural retina. Furthermore, there was no study reported on the degradation of these scaffolds, and no mention of the byproducts of this degradation and their toxicity. Nevertheless, their design was promising, taking the concept from Redenti *et al.* (2009) and developing it further [94].

The follow-up publication to Jung 2018 had a modified channel geometry, with the ‘wine glass’ shape replaced with an ‘ice cube tray’ structure [99] Each channel consisted of a larger square reservoir, with 9 holes at the bottom. The intention of the scaffold redesign was to increase the volume of each channel, and consequently the number of cells that can be cultured in the scaffold. Unlike the previous design, the holes were not meant for axonal growth, but nutrient, waste, and fluid transfer. HPSC derived photoreceptors were seeded on the scaffold, pre-coated with laminin, for 5 days. To assess polarisation of the cells, markers for both outer segments (peripherin) and presynaptic markers (vesicular glutamate transporter 1) were stained and analysed. Unexpectedly, the markers for outer segments were located on the bottom of the scaffold, near the holes, and the markers for presynaptic markers were located near the top of the scaffold. This photoreceptor polarity was the opposite of the one observed in the previous ‘wine glass’

scaffolds but could be attributed to the shorter through-holes, or the larger clustering of the cells in the channels [99].

All of the papers discussed thus far have used photolithography to fabricate patterned scaffolds, as it is a commonly used method to produce patterns with high resolution. An alternative technique is to fabricate patterned scaffolds with a high-resolution 3D printer. The Tucker group used the Nanoscribe, a two-photon polymerisation system, to print patterned scaffolds for alignment of human induced pluripotent stem cell (iPSC) derived photoreceptors [100]–[102]. Their scaffolds were designed to have vertical channels that extend throughout the structure, in a hexagonally packed array, with a range of diameters (15, 20 and 25 μm), as seen in Figure 1.13. Additionally, horizontal interconnected pores (with a diameter of 7 μm) were added to the design to facilitate diffusion through the scaffold.

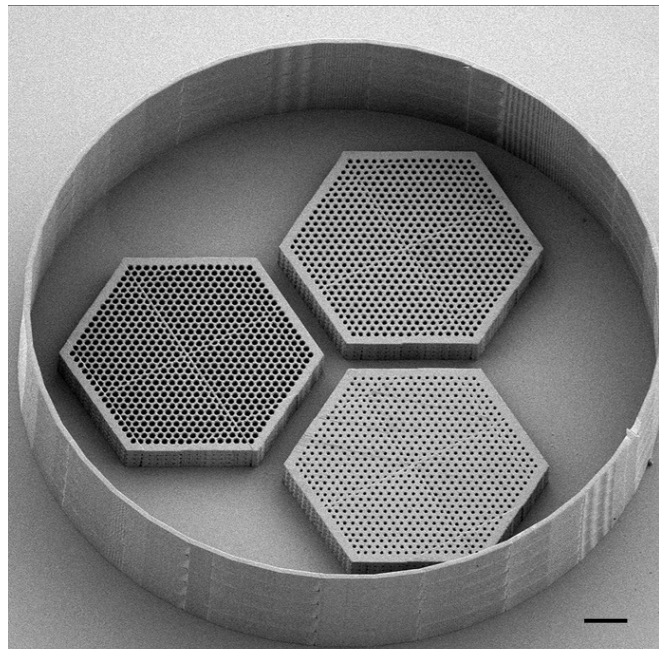


Figure 1.13: Scaffolds for retinal cell cultures. Scaffolds were 3D printed on the Nanoscribe with various hole diameters (15, 20, and 25 μm) packed into a hexagonal array. Scale bar: 200 μm . Figure adapted with permission from [100].

Their initial tests were conducted on scaffolds printed with the IP-S resin, a biocompatible material produced by the Nanoscribe and optimised for the printer. Retinal progenitor cells were seeded on the scaffolds, coated with laminin, and incubated for 2 days. In all three scaffolds, the cells migrated into the scaffold and extended processes in the same direction as the channels, with the 25 μm diameter scaffold exhibiting the highest number of cells integrated within the scaffold [100].

With these promising results, further studies were carried out with the same geometry, but with custom resins, synthesised from both synthetic (PCL) and natural polymers. These resins required extensive optimisation to determine the printing parameters that achieve the required resolution, including scanning speed, laser power, slicing and hatching distances, as well as the composition of the resin [101], [102]. The PCL printed scaffolds showed promising *in vitro* results when seeded with retinal progenitor cells, as well as no adverse outcome post-transplantation in porcine eyes [101]. The natural polymer resins chosen were collagen, gelatin, hyaluronic acid, and a 50/50 mixture of gelatin and hyaluronic acid, all methacrylated. When seeded with retinal progenitor cells, the 50/50 blend of gelatin and hyaluronic acid had the highest cell viability [102].

1.5 Conclusion

Irreversible loss of photoreceptors, due to AMD or IRD, is one of the main causes for retinal degenerative diseases. This results in visual impairment and can even cause blindness. Treatment methods to halt the progression of the disease exist, including anti-VEGF injections [8] and some gene therapy treatments [24]. However, these treatments are limited, and do not offer a cure to restore the damaged vision. Other treatments like prosthesis do offer some vision restoration, however, yield low spatial resolution [35].

Photoreceptor replacement therapies have been studied extensively, with promising results reported over the last few decades [39], [45], [47], [48], [54], [57], [71]. These studies aim to deliver healthy photoreceptor cells to the damaged retina to replace those

lost through degeneration, however, findings by Pearson *et al.* have call into question the reported results [73]. More recent studies have established that using biomaterial scaffolds to deliver cells can increase the survival and integration of the transplanted cells in the host retina [40], [42], [86], [90], [92].

Patterned scaffolds were developed to polarize photoreceptors *in vitro* and improve the likelihood of forming synaptic connection with the host cell once transplanted, overcoming some of the limitations of using non-patterned scaffolds. Different patterns have been studied, and photoreceptor cell responses to the patterns suggest a possibility of aligning cells in the scaffolds [94], [95], [97], [100]–[102].

Jung *et al.* (2018) demonstrated clear control over photoreceptor polarisation within a patterned scaffold. Their design was the most promising and state of the art out of the literature that had been published, as it was the closest to the native retinal photoreceptor organisation. However, their design did have limitations, primarily in the density of photoreceptors that can be seeded in the scaffold as well as their choice of material. The work of Jung *et al.* (2018) inspired the micro-patterned hydrogel geometry developed in this thesis, which is focused on improving their design and overcoming its limitations [98]. This will be discussed in detail in Chapter 2.

Chapter 2: Scaffold fabrication: material synthesis, scaffold designs and micro-fabrication techniques

The success of Jung *et al.* (2018) in fabricating a micro-patterned scaffold for polarisation of photoreceptor cells motivated the work presented in this thesis. Their unique scaffold design showed promising photoreceptor alignment, and the presence of pre-synaptic markers at the bottom of their channels suggested that the photoreceptor cells were primed for synaptic formation, which had not been shown in any of the other publications [98]. This project aims to take inspiration from their design and build on it.

This chapter provides a description of the materials used in this project, as well as their synthesis and validation protocols. Additionally, two scaffold geometries, which include a ‘wine glass’ channel design, inspired by Jung 2018, and a ‘champagne flute’ channel design, will be fabricated using two different techniques. Finally, some initial demoulding attempts will be described.

2.1 Material synthesis

2.1.1 Gelatin Methacryloyl

2.1.1.1 Background

Gelatin is a natural material derived from the hydrolytic degradation of collagen [103], [104]. It has been a popular biomaterial used in the field of bioengineering due to its biodegradability and biocompatibility. It can be extracted from several different animal sources [105], most commonly porcine [106], fish [107] and bovine tissues [108]. Gelatin contains many arginine-glycine-aspartic acid (RGD) sequences that facilitate cell binding through interactions with integrin, promoting cell attachment [109], [110].

Gelatin methacryloyl (GelMA) hydrogels have been widely used as biomaterials in the field of tissue engineering as they possess some of the same properties as gelatin hydrogels, including biocompatibility, biodegradability, and cell binding sequences that enhance cellular attachment. However, unlike gelatin, they are chemically crosslinked making them more stable and making their mechanical properties more tuneable. This allows researchers to match the hydrogel properties to the various tissues investigated, making it a favourable material choice for a wider range of tissue engineering applications. The hydrogel crosslinking mechanism is achieved through radical polymerisation; a small amount of initiator is added to the precursor solution in order to generate sufficient free radicals for the polymerisation to occur after exposure to UV irradiation, leading to a kinetic reaction chain. This crosslinking mechanism makes GelMA a versatile material, allowing the hydrogels to be 3D printed or moulded.

One of the first GelMA papers published was Van Den Bulcke et al. (2000) [104]. The authors studied the rheological properties of crosslinked hydrogels, and the effect of varying the degrees of substitution, as well as polymer and initiator concentrations. Since then, several different applications of this material were reported [111]. These included regeneration of the cornea [112], skin [113], bone [114], [115], cartilage [116], tendon [117], and vasculature [118].

2.1.1.2 Synthesis of gelatin methacryloyl

Methacryloyl gelatin (GelMA) was synthesized using modified versions of the protocols described by Ouyang 2020 and Loessner 2016 [119], [120]. Briefly, a 10% (w/v) porcine gelatin solution was prepared by dissolving 5 g of gelatin in 50 mL of milliQ water in a 150 mL round bottom flask. To fully dissolve the gelatin, the mixture was heated to 50°C in a water bath, until the mixture became transparent and homogenous. To the gelatin solution was then added 2 mL/g of methacrylic anhydride (Sigma-Aldrich) and the mixture was left to react for 2 to 3 hours at 50°C. In order to control the degree of methacryloyl functionalization, the solution can be left to stir for longer (increasing the

degree of functionalization), and the concentration of methacrylic anhydride added can be varied (higher concentration increases the degree of functionalization).

When the reaction was complete, the solution was transferred into 50 mL falcon tubes and centrifuged at 3500 g for 3 minutes at room temperature in order to remove the unreacted methacrylic anhydride. The supernatant, containing the GelMA, was decanted into a beaker and then, diluted with water at 40 °C (twice the volume of the GelMA solution). The solution was then transferred into 12 kDa molecular weight cut off dialysis tubing and dialysed against DI water for 3 days at 40°C, while changing the water three times a day. After dialysis, the GelMA solution was neutralised to pH 7.4, filtered, frozen at -80°C and lyophilised.

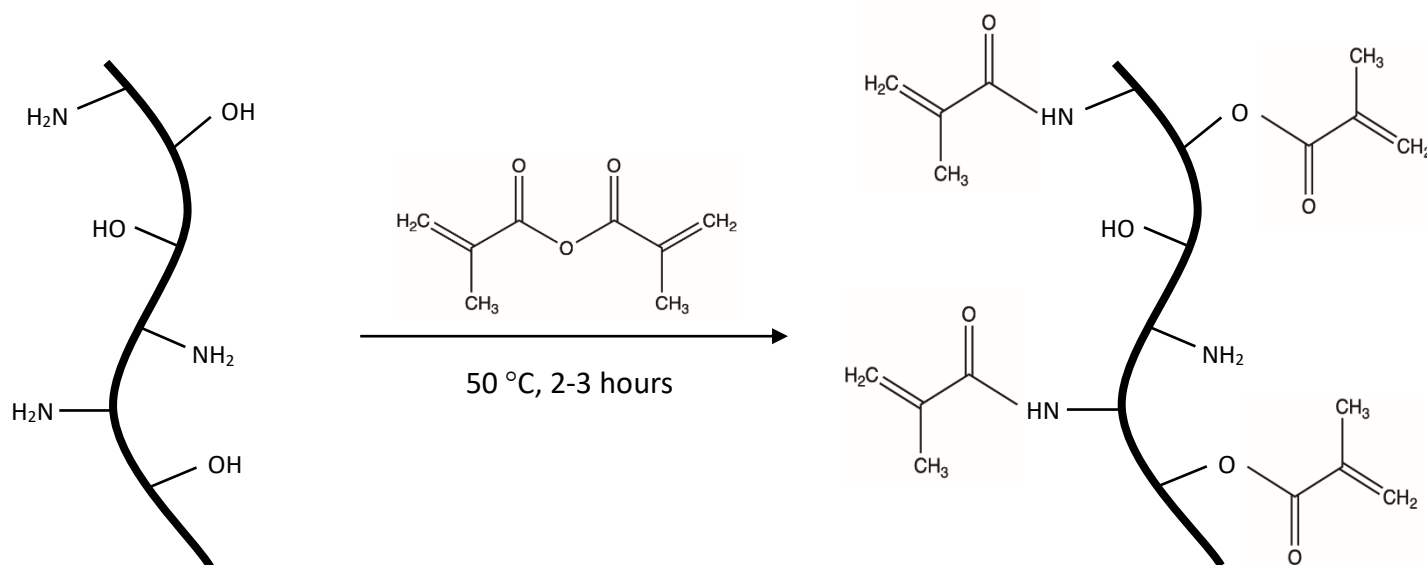


Figure 2.1: Chemical reaction of the methacrylation of gelatin. The gelatin reacts with the methacrylic anhydride to form a methacryloyl gelatin.

The degree of functionalization (DoF) of GelMA was then determined using a fuoraldehyde assay [120]. The synthesised GelMA was dissolved in 1X PBS as a concentration of 0.5 mg/mL, and gelatin was dissolved in 1X PBS at several concentrations (0.05, 0.1, 0.5, 1 mg/mL) to generate a standard curve. All solutions were warmed up at 50°C for 30 minutes and mixed until the polymers are fully dissolved. The fuoraldehyde reagent solution (Thermo Scientific™ Fluoraldehyde™ o-Phthaldialdehyde Reagent Solution) was warmed up to room temperature. This reagent reacts with the primary amines on the polymer and produces a quantifiable fluorescent signal that is linear over a wide range of concentrations. For the assay, 300 µL of each of the GelMA and gelatin solutions were mixed with 600 µL of the fuoraldehyde reagent in separate Eppendorf tubes, in addition to a PBS-Fluoraldehyde control solution. Once mixed homogeneously, 250 µL triplicates of each solution were pipetted into a 96-well plate and the fluorescence intensity was read at 450 nm after exciting the plate at 360 nm (plate reader: Spectra Max M5). In order to determine the DoF, the average fluorescence reading of the PBS control was subtracted from the average fluorescence readings of the three replicates for each concentration, and these values were plotted in function of the concentration of the polymer. A linear trendline was plotted to determine the slope of the curve of the gelatin measurements. The DoF of the GelMA solution was calculated using the following equations:

$$DoF = \frac{0.5 \text{ mg/mL} - UC \text{ mg/mL}}{0.5 \text{ mg/mL}} \times 100\%$$

$$UC \text{ mg/mL} = \text{unfunctionalised concentration} = \frac{(\text{average reading} - \text{control reading})}{\text{slope}}$$

Plugging in the values into these equations, the calculated DoF of the synthesised GelMA $\approx 55\%$

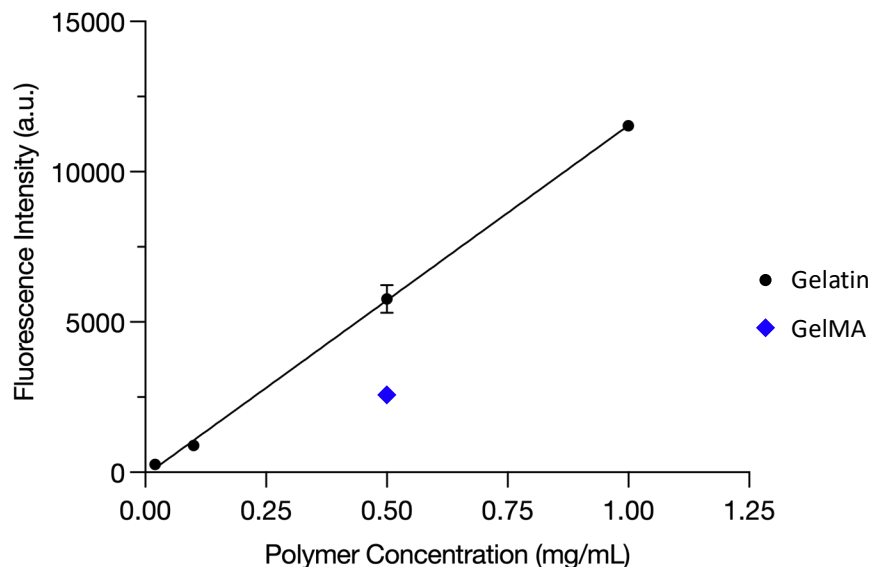


Figure 2.2: Determination of degree of functionalisation of GelMA. Standard curve of fluorescence intensity as a function of gelatin concentration, to determine the DoF of GelMA. The data was generated by Dr. Jonathan Wojciechowski (n=3).

2.1.2 Hyaluronic acid methacryloyl

2.1.2.1 Background

Hyaluronic acid (HA), also known as hyaluronan, is a natural polymer that can be found throughout the human body, most commonly in the extracellular matrix of tissues [121]. HA is also one of the main components of retinal extracellular matrix, which makes it an obvious option for use in tissue engineering in the eye [122]. HA is a linear polysaccharide, consisting of alternating units of two types of saccharides: D-glucuronic acid and 3-*N*-acetyl-D-glucosamine [123], [124]. Additionally, HA is a non-sulfated hydrophilic glycosaminoglycan [124]–[126].

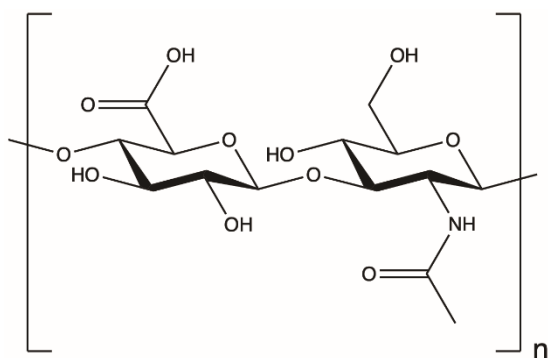


Figure 2.3: Chemical structure of hyaluronic acid.

HA is one of the main components of the ECM and plays a crucial role in cell proliferation and migration, cellular signalling, as well as wound healing and reduction of long-term inflammation [123], [127]–[129]. It has a rapid turnover time in the body due to the action of hyaluronidase [123], [130]. HA has been used widely in the medical field for decades [131] and was first reported as a material isolated from bovine vitreous in 1934 [132]. More recently, it has been used for the fabrication of biomaterials for tissue engineering purposes [133], [134], including the treatment of retinal diseases, as described in Chapter 1. The Young group encapsulated retinal progenitor cells into HA-based hydrogels and transplanted the scaffolds into mouse retinas. Three weeks post transplantation, the cells were able to integrate, differentiate and expressed markers for mature photoreceptors, and the hydrogels were fully degraded [89]. The Shoichet group also focused on using HA-based hydrogels as retinal scaffolds. Their research demonstrated that using hyaluronan-based hydrogel to transplant rod photoreceptors into the subretinal space improved their survival and integration when compared to those injected in a saline solution. They attributed these results to the interactions of a cell adhesion receptor (CD44) with the hyaluronic acid [40], [42]. A further study from this group showed that HA-based hydrogels also increase photoreceptor survival and maturation *in vitro* significantly, as seen in Figure 1.8 [90]. The positive results from these papers make HA a promising choice of material for the purpose of this thesis.

Several chemical modifications of HA have been tested, due to the three functional groups it presents: the carboxylic acid, the N-acetyl group and the two hydroxyl groups. Acrylates and methacrylates are the most common reactive groups that have been used for radical polymerisation as they react rapidly with radicals. Additionally, HA has a backbone that presents modifiable carboxyl groups on the D-glucuronic acid unit and hydroxyl groups of the alcohols at C-6 on the *N*-acetyl-D-glucosamine unit [135].

These modified HA hydrogels have been used for bone regeneration [136]–[139], cartilage repair [140], cardiac valve tissue engineering [141], [142], neural progenitor cell differentiation [143], tumour targeted drug delivery [144], cell encapsulation [145], and as surgical glue [129].

One of the most common modifications of HA is reacting it with a methacrylic anhydride to form a methacrylated HA or HA methacrylate (HAMA) through an esterification of the alcohol group [124]. This reaction can be performed in either organic solvents or in an aqueous environment [146], and can yield a range of degrees of functionalisation [124], [135], [146]. The first papers referring to this reaction include Smeds 1999 and 2001 [147], [148].

HAMA is a semi-synthetic and photocrosslinkable hydrogel that is widely used in the field of tissue engineering due to its biocompatibility and ease of crosslinking. The hydrogel crosslinking mechanism is through radical polymerisation, similarly to GelMA. This modification produces hydrogels that have more robust mechanical properties when compared to simple HA hydrogels without compromising the material biocompatibility [124], [149].

2.1.2.2 Synthesis of hyaluronic acid methacryloyl

Hyaluronic acid methacrylate (HAMA) was synthesized using a modified version of the protocol described in Ouyang 2020 [119]. Briefly, a 1% (w/v) hyaluronic acid solution

was prepared by dissolving 500 mg of sodium hyaluronate (research grade 100 kDa sodium hyaluronate, Lifecore Medical) in 50 mL of 0.25 M carbonate buffer pH 10, at room temperature, in a 150 mL round bottom flask. Once fully dissolved, the pH of the solution was equilibrated to 10, after which the flask was placed into a saltwater ice bath. 2.5 mL/g of methacrylic anhydride was then added dropwise to the solution (in excess) and the mixture was left to react overnight. Afterwards, the solution was neutralised to pH 7, filtered and filled in 6-8 kDa molecular weight cut off dialysis tubing and dialysed against DI water for 3 days at room temperature, while changing the water three times a day. After dialysis, the HAMA solution was frozen at -80°C and lyophilised. The degree of methacrylation (number of methacrylate groups per 100 disaccharide units) [124] of the HAMA can be controlled through the concentration of methacrylic anhydride added, the solvents chosen, as well as the reaction time, allowing a degree ranging from 5 to 49% [135], [146].

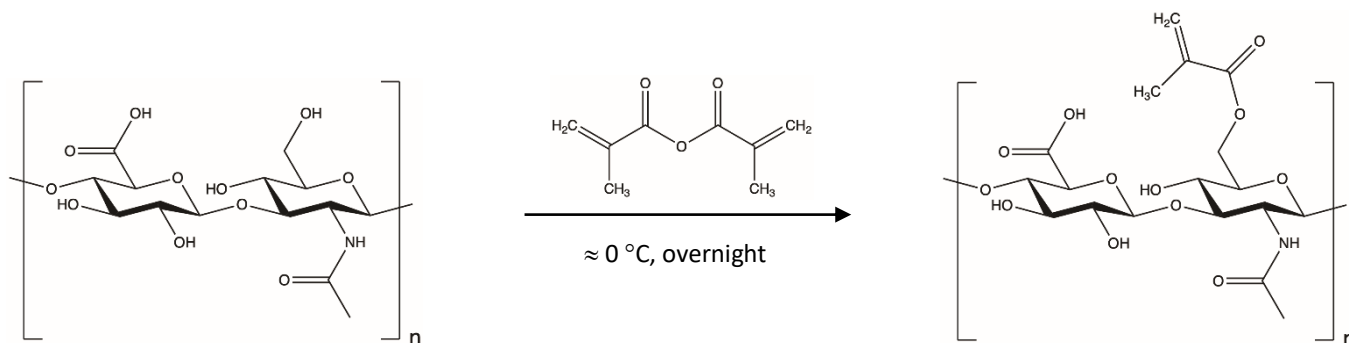


Figure 2.4: Chemical reaction of the methacrylation of hyaluronic acid. The hyaluronic acid reacts with the methacrylic anhydride to form hyaluronic acid methacryloyl.

The degree of functionalization of the HAMA was determined by ^1H nuclear magnetic resonance (NMR) spectroscopy. To acquire the NMR spectra, 10 mg of HAMA was dissolved in 600 μL of deuterium oxide containing 1 mg/mL 3-(trimethylsilyl)propionic-2,2,3,3- d_4 acid sodium salt (TMSP) and the solution was measured using a spectrometer at a frequency of 400 MHz (JEOL JNM-ECZ400S/L1). Tetramethylsilylpropanoic acid

(TMSP) was used as an internal reference at 1 mg/mL because it has a sharp chemical shift at a low resonance frequency that does not interfere with the resonance of the other protons in the polymer [150]. The NMR measurements were run by a postdoc in the group, Dr. Jonathan Wojciechowski. The calculations were based on previously reported literature, with the assumption that the reaction causes a substitution at the 6-position [119], [126]. A representative ^1H -NMR spectrum of HAMA is analysed in Figure 2.5 below. Phase correction was applied to the spectrum to obtain the absorptive peaks, the baseline was corrected, and the chemical shift was adjusted to the TMSP signal at 0 ppm.

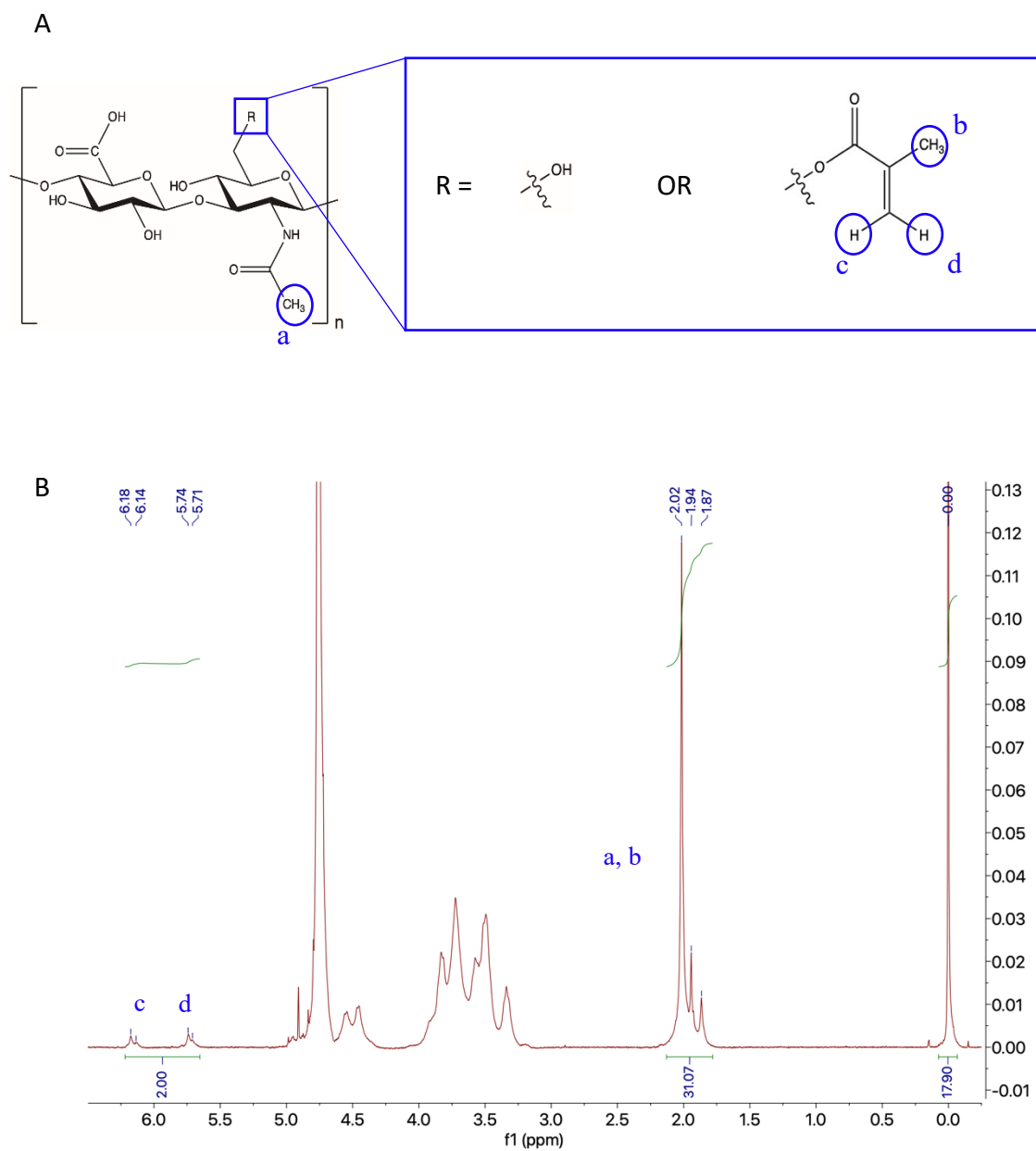


Figure 2.5: Determination of degree of functionalisation of HAMA. (A): Chemical structure for HAMA specifying the proton locations for the ^1H -NMR spectrum. (B): ^1H -NMR spectrum for HAMA. NMR measurements were generated by Dr. Jonathan Wojciechowski.

The integrals of a (acetyl-CH₃) and b (methacrylate-CH₃) overlap in the NMR spectra at around 2 ppm, while c and d (methacrylic vinyl protons) are adjacent between 5.5 and 6.5 ppm. If c + d = 2 and b = 3, the measured integration of a is equal to 28.07. When the polymer has a degree of functionalisation of 100%, the ratio of (c + d):a = 2:3, meaning a would be equal to 3. As that is not the actual value of a, the real value of the integration of c + d is equal to 0.214. From there, the degree of functionalisation of the polymer is:

$$\frac{c + d}{2} \times 100\% = \frac{0.214}{2} \times 100\% = 10.7\% \approx 11\%$$

In addition, the degree of methacrylation was calculated in mmol/g using TMSP as an internal standard based on the following formula from Claaßen 2018 [151]:

$$DM = \frac{\int \text{methacryl}}{\int \text{TMSP}} \times \frac{9H}{1H} \times \frac{n \text{ (TMSP) [mmol]}}{m \text{ (HA derivative) [g]}}$$

Taking the average of three synthesised batches, the degree of methacrylation of HAMA is 0.318 mmol/g $\pm$ 0.11 mmol/g.

2.1.2.3 Cy5 functionalisation of HAMA

Cy5 is a far-red fluorescent label traditionally used for imaging and flow cytometry. It is commonly chosen due to its stability and resistance to photobleaching, low toxicity and near-infrared fluorescence signal, making it ideal for imaging [152]. Here, cy5 is functionalised to HAMA to label the hydrogel for imaging during cell culture and following transplantation.

A 1% (w/v) HAMA solution was prepared by dissolving 50 mg of HAMA in 5 mL of 0.1 M MES buffer at a pH of 6.5. EDC (EDC hydrochloride, Fluorochem) and NHS (N-Hydroxysulfosuccinimide sodium, Sigma-Aldrich) were added at 26 equivalents (2.49

mg) and 20 equivalents (1.15 mg) respectively relative to 1 equivalent of HAMA. The mixture was left stirring at room temperature for 20 minutes in the dark, after which the cy5-amine dye (cyanine5 amine dye, Abcam) was added at 20 equivalents (6.54 mg) and the reaction buffer was left to stir overnight at room temperature. The following day, the solution was filtered against DI water using 10 kDa MWCO Amicon filter repeatedly until the colour of the flow through liquid was clear. The cy5 functionalised HAMA solution was frozen at -80°C and lyophilized. To verify that the Cy5-HAMA was successfully functionalised, UV-vis (absorbance) and fluorescence spectra were measured for both the Cy5-HAMA and non-functionalised HAMA at a concentration of 0.05% (w/v), and the plots can be seen in Figure 2.6. The absorbance was measured between 350 and 750 nm, and the fluorescence was measured with an excitation peak at 650 nm and an emission peak at 670 nm. Both absorbance and fluorescence peaks are present in the functionalised Cy5-HAMA, and absent in the HAMA, indicating a successful reaction. These measurements were conducted by Brian Chen, a PhD student in the group.

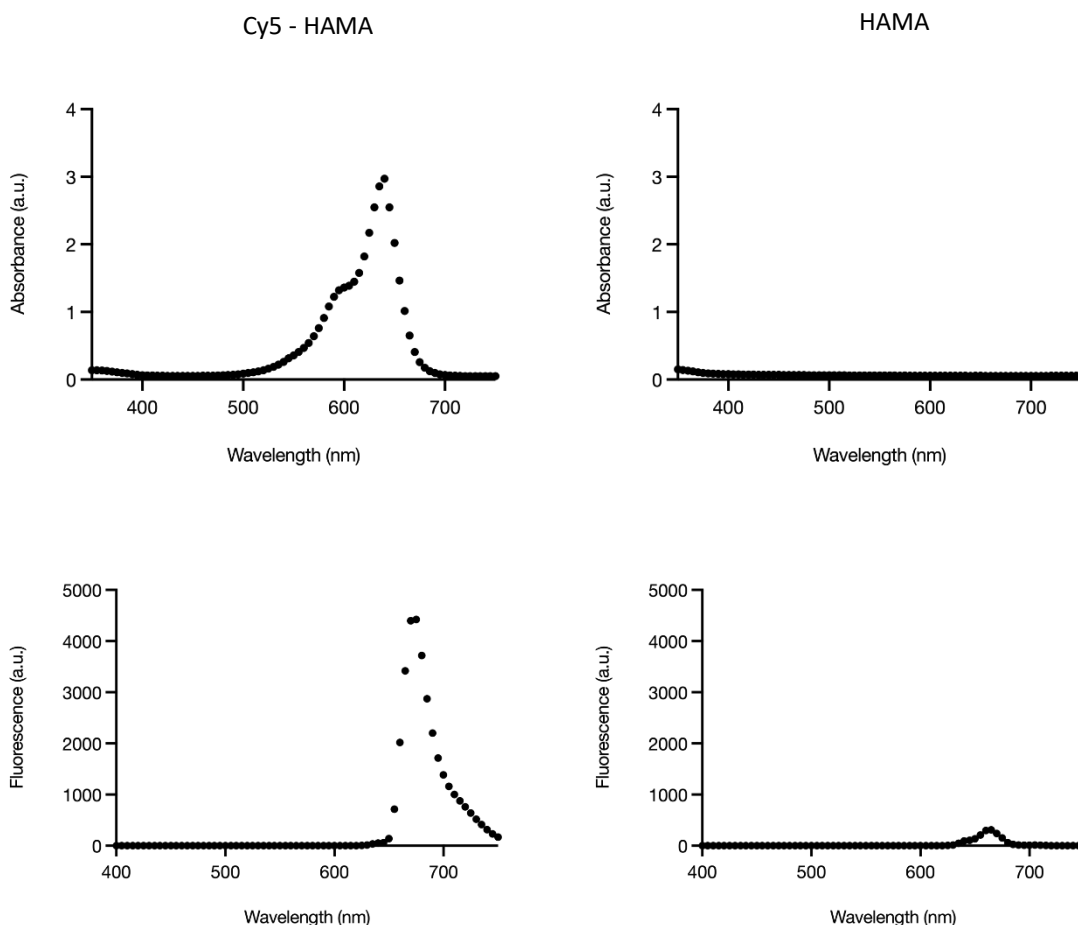


Figure 2.6: Absorbance and fluorescence plots for HAMA-Cy5 and HAMA. The absorbance was measured between 350 and 750 nm, and the fluorescence was measured with an excitation peak at 650 nm and an emission peak at 670 nm. Data was generated by Brian Chen (n=3).

2.1.3 Poly(ethylene glycol) dimethacrylate

2.1.3.1 Background

Poly (ethylene glycol) (PEG) is a water soluble hydrophilic polymer that has been widely used in the field of regenerative medicine for a variety of biomedical applications, due to its biocompatibility and versatility [153]. These applications include controlled release of drugs and other types of molecules, as well as vehicles for the delivery of cells and scaffolds for tissue engineering and regeneration [154]. Furthermore, the chemical and structural properties of the PEG hydrogel components can be controlled, enabling fine-tuning of the hydrogels according to the application [155]. This can be achieved by varying different properties, including the molecular weight of the PEG, the ratio of PEG to crosslinker and type of crosslinker.

PEG polymers can be end-functionalised with different chemical groups that determine their crosslinking method [156]. This includes substitution of the terminal hydroxyl groups of PEG with a methacrylic anhydride to form a PEG dimethacrylate (PEGDMA) [157]. PEG polymers have also been used along with GelMA [158] and HAMA [141], [159]–[161] to produce more tuneable hydrogels with a wider range of mechanical properties and cell binding properties [158], as PEG on its own does not support cell binding[153].

2.1.3.2 Synthesis of polyethylene glycol dimethacrylate

A 20% w/v PEG solution was prepared by dissolving 2 g of 6 kDa PEG (Sigma-Aldrich) in 10 mL of anhydrous dichloromethane. Methacrylic anhydride and triethylamine were both added at 4 equivalents relative to the 1 equivalent of PEG. The mixture was sealed

and left to stir at room temperature for 3 days, after which nitrogen gas was used to evaporate half of the volume of dichloromethane from the solution. The remaining volume was added dropwise into a falcon tube containing 40 mL of a cold solution of diethyl ether to precipitate the reacted PEG. The tube was then centrifuged at 7000 RCF for 5 minutes at -4°C, after which the supernatant was removed, and the pellet was dried by nitrogen. The PEG was re-dissolved in 10 mL of dichloromethane and the process of precipitation and centrifugation was repeated twice more.

The degree of functionalization of the PEGDMA was determined by ^1H nuclear magnetic resonance (NMR) spectroscopy. To acquire the NMR spectra, 10 mg of PEGDMA was dissolved in 600 μL of deuterium oxide and the solution was measured using a spectrometer at a frequency of 400 MHz (JEOL JNM-ECZ400S/L1). The NMR measurements were run by a PhD student in the group, Lisha Zhong. The calculations were based on previously reported literature [162]. A representative ^1H -NMR spectrum of PEGDMA is analysed in Figure 2.7 below. Phase correction was applied to the spectrum to obtain the absorptive peaks, the baseline was corrected, and the chemical shift was adjusted to the signal at 0 ppm.

When the polymer has a degree of functionalisation of 100%, the ratio of $(b + c):e = 2:1$. As the value of e is fixed at 2, the value of $b + c$ should be equal to 4. However, $b + c = 0.73 + 1.17 = 1.9$.

$$\frac{b + c}{4} \times 100\% = \frac{1.9}{4} \times 100\% = 47.5\%$$

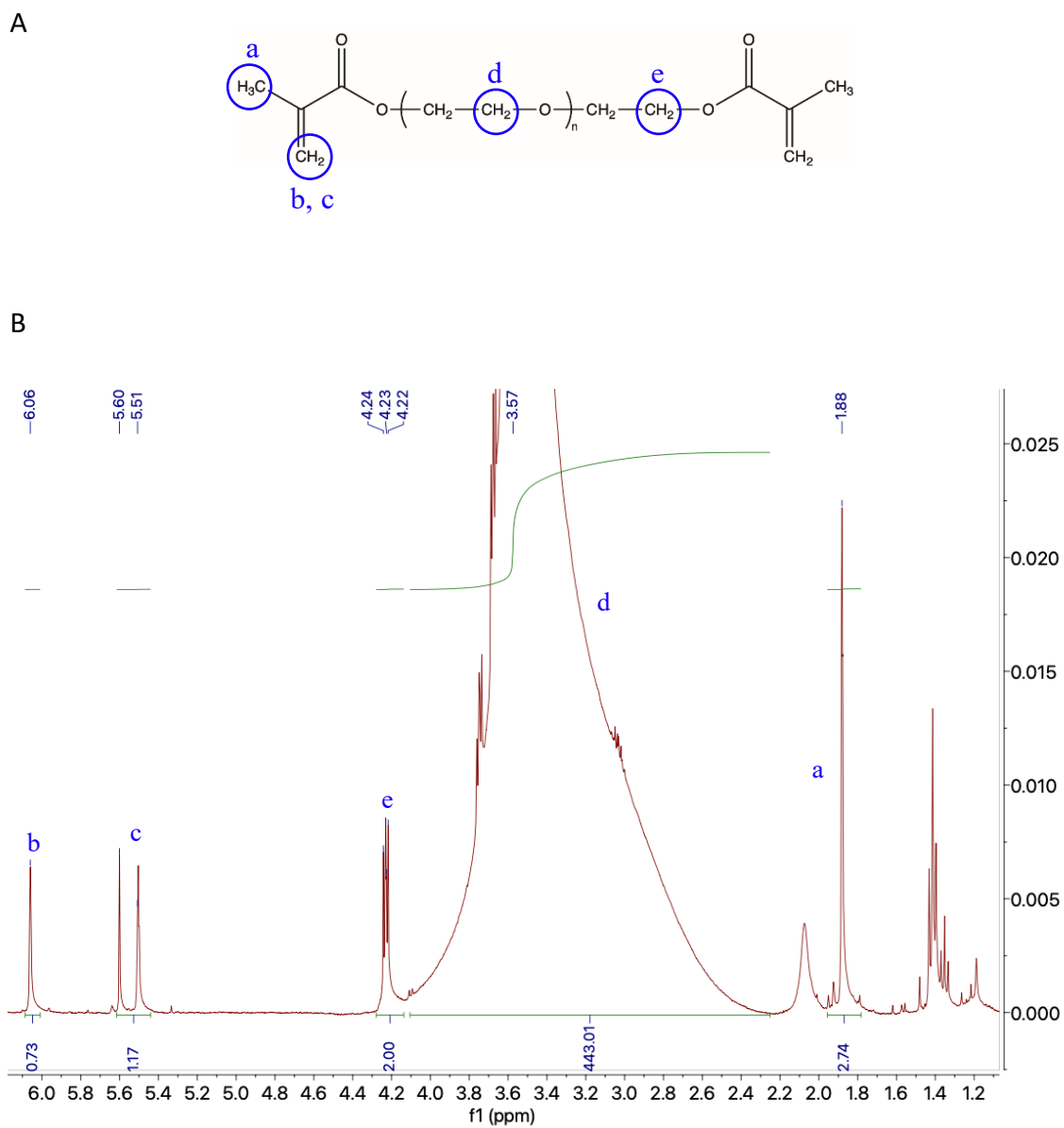


Figure 2.7: Determination of degree of functionalisation of PEGDMA. (A): Chemical structure for PEGDMA specifying the proton locations for the ^1H -NMR spectrum. (B): ^1H -NMR spectrum for PEGDMA. NMR measurements were generated by Lisha Zhong.

2.1.4 Crosslinking mechanism

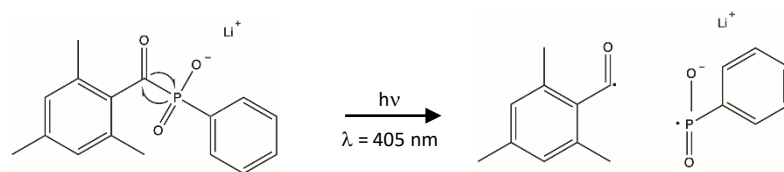
The polymers synthesised in this section can be photo-crosslinked, with spatial and temporal control [163] to form hydrogels. These photopolymerisable hydrogels have been highly investigated in the literature for tissue engineering applications [153], [164].

Photopolymerization is a technique that uses light, either from the visible spectrum or ultraviolet (UV), to crosslink a material, in the presence of a photoinitiator. For the experiments described in this thesis, the choice of photoinitiator was Lithium phenyl-2,4,6-trimethylbenzoylphosphine oxide (LAP, Sigma Aldrich). LAP was chosen instead of Irgacure 2959 (commonly used in the literature for cell encapsulation within hydrogels) due to some evidence of higher cell viability with LAP [165].

The hydrogels are crosslinked through a free radical polymerisation mechanism. This mechanism is formed of three steps (initiation, propagation, and termination), and can be seen in Figure 2.8. Following photon absorption, radical photoinitiation begins with the LAP photoinitiator being cleaved into two dissociated free radicals, that can initiate polymerization. The radicals then transfer to the methacrylate groups of the polymer, which in turn start crosslinking with other methacrylate groups, propagating the radicals. Finally, the radical terminates and ends crosslinking by crosslinking to either an initiator, a methacrylate group with a radical, or an O_2 molecule.

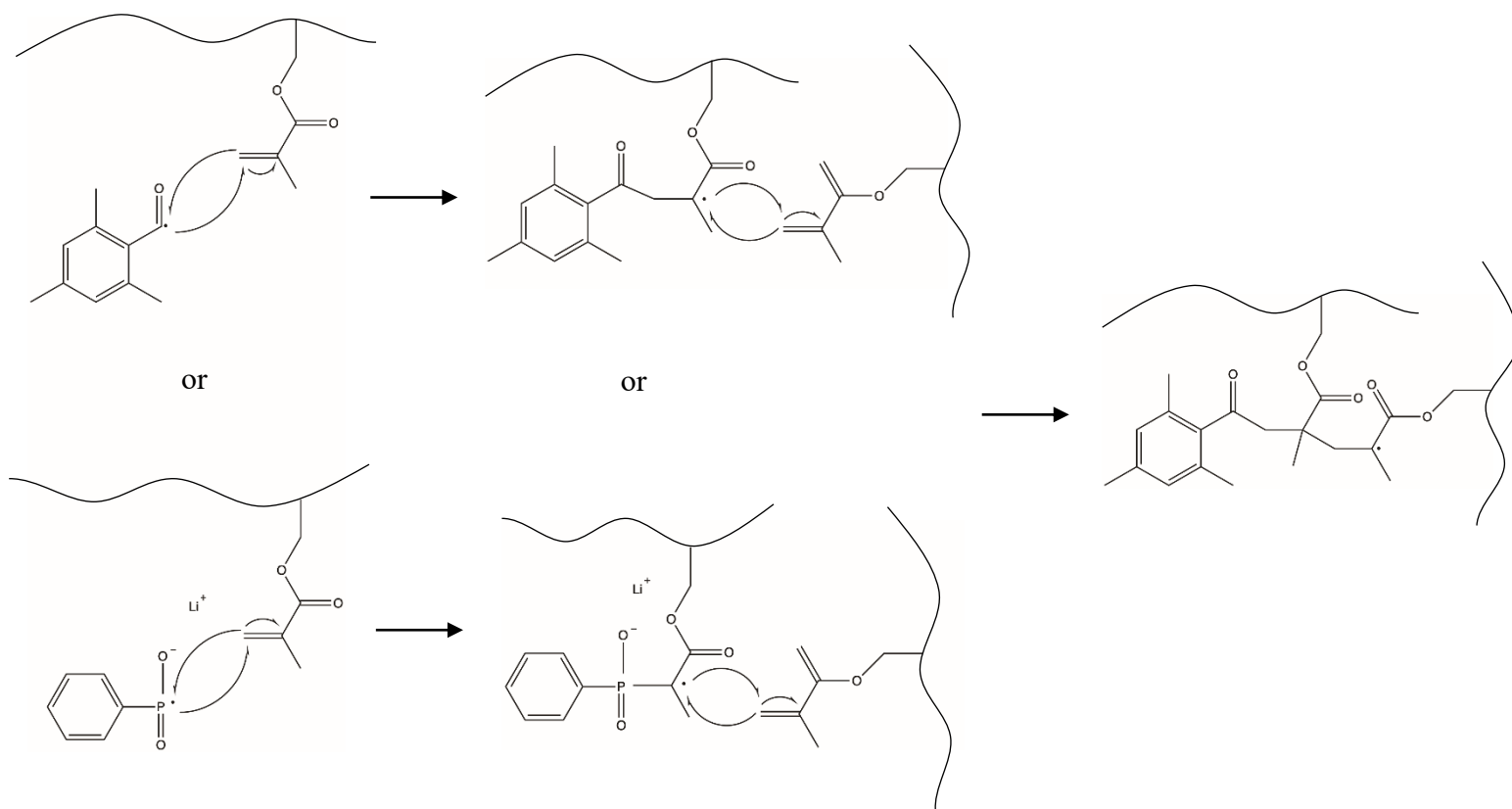
Figure 2.8: Free radical polymerization mechanism. The polymerization uses LAP as a photoinitiator, and includes initiation, propagation, and termination steps [153], [163]. The figure can be seen on the next page.

Initiation

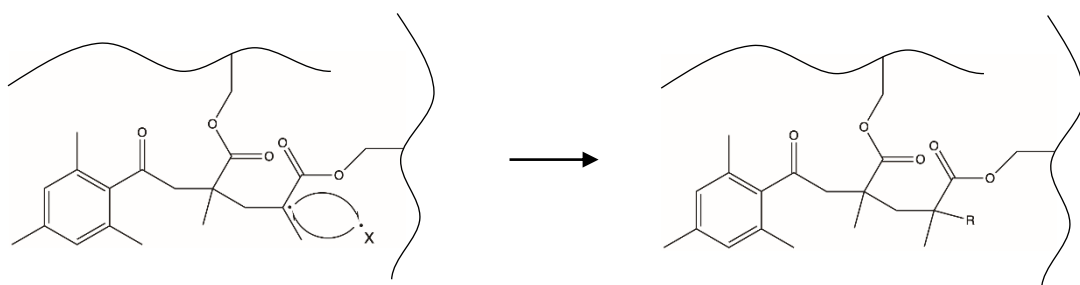


LAP (photoinitiator)

Propagation



Termination



2.2 Scaffold design 1: ‘Wine glass’ channels scaffold

As described in Chapter 1, Jung *et al.* (2018) were successful in fabricating a 3D micropatterned scaffold for the purpose of guiding photoreceptor axonal growth to polarise the cells [98]. Their design was promising, and the closest to the native retinal photoreceptor organisation that had been published. In their model design, each channel could hold up to three photoreceptor cell bodies as illustrated in Figure 2.9. They fabricated their scaffolds with both PDMS and PGS, the latter being a biocompatible and degradable polymer. Their choice of PGS was primarily due to its popular use in microfabrication because of its adjustable mechanical properties and success in micropatterned scaffolds [166]–[170], including some publications using it for retinal cell culture [94], [98], [166]. However, PGS is a completely synthetic material, and required the addition of cell binding proteins such as laminin and fibronectin for retinal cells to adhere to the hydrogels [94], [98]. Therefore, a natural polymer, here GelMA, was chosen instead for the experiments described in this section. This material was chosen due to its RGD peptide sequence that facilitate cell binding through interactions with integrin, as mentioned in section 2.1.

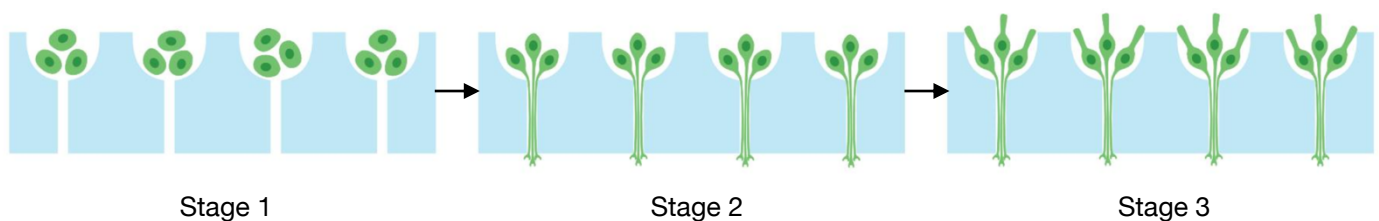


Figure 2.9: ‘Wine glass’ channels scaffold. The scaffold contains photoreceptor cells with morphological predictions at the different stages of culture. Stage 1: Photoreceptor cell bodies lose their outer segments and axons following sorting. Stage 2: Photoreceptor cells begin forming axons that grow into the stems of the scaffold. Stage 3: Photoreceptor cells being growing outer segments.

2.2.1 Silicon master mould fabrication

The promising results reported in Jung *et al.*'s 2018 paper motivated the initial designs in this project [98]. The fabrication protocol was described in their paper as well as their supplementary documents and was replicated by Dr. Hyejeong Seong, a postdoc in the Stevens group. The mould was fabricated using photolithography, which is a process used to form micro-patterns into a silicon substrate, also called a wafer. Ultraviolet light is used in addition to an optical photomask to transfer the geometric pattern from the mask to the substrate coated with a light sensitive photoresist.

The array of channels was fabricated on a 4-inch silicon wafer (purchased from University Wafers) using a 5" low reflective chrome photomask (purchased from Photo Data). The master mould was fabricated via photolithography and dry etching process. The dry etching consisted of a two-step process: an isotropic etching to form the wider top reservoir of the scaffold, followed by an anisotropic etching.

The isotropic etching step used to form the upper chamber was divided into two phases, both performed with a reactive ion etcher (RIE). The first consisted of a 25-minute chemical reaction-based etching process, at a pressure of 16 mTorr and a plasma power of 50 W, to allow lateral expansion of the Si. This reaction laterally etched the silicon just beneath the photoresist, with minimal vertical etching, to create a larger diameter opening for the top of the channels. The second step was a 5-minute etching process using the same RIE setup, but at a pressure of 6 mTorr and a plasma power of 60 W. This second etching step was performed to both horizontally and vertically etch the silicon, creating the cup like shape of the upper channel.

The anisotropic etching was used to create the full vertical holes and was performed with a deep reactive ion etcher (DRIE). Each DRIE cycle consisted of (i) 130 sccm (standard cubic centimetres per minute) of SF₆ gas and 6 sccm of O₂ gas with a process pressure of 15 mTorr and power of 800 W for a 9 second etch phase, and (ii) 85 sccm of

C4F8 gas with a process pressure of 14 mTorr and power of 600 W for a deposition phase of 6.5 seconds. 100 cycles were conducted to achieve a height of 15 to 20 μm . The master mould was cleaned with acetone and DI water before the negative mould fabrication. Several values for diameter (D) and distance (d) (Figure 2.10 (B)) were chosen to show that this method can be used for a variety of dimensions, and accordingly fabricated. Representative scanning electron microscopy images of some of the conditions are shown in Figure 2.11.

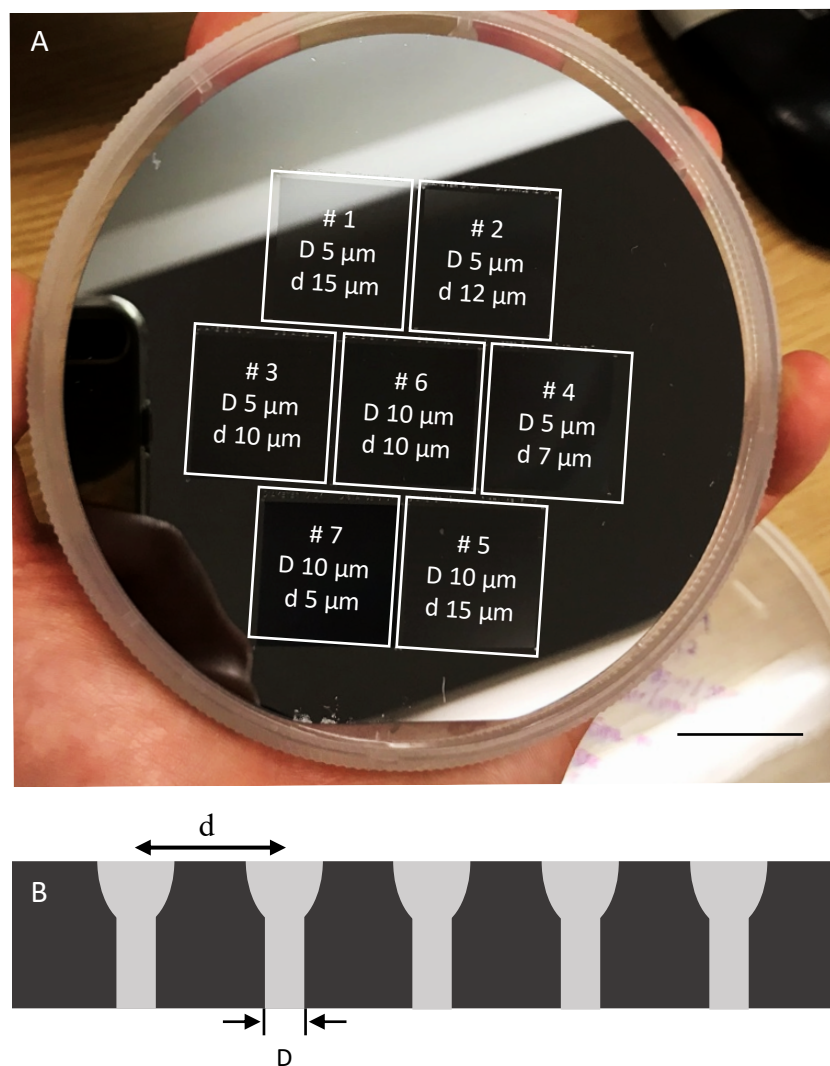


Figure 2.10: Silicon master mould. (A) The mould was fabricated with the corresponding values of diameter and distance by Dr. Hyejeong Seong. (B) Illustration of the cross section of the mould. Scale bar 2 cm.

Looking at the scanning electron microscopy images in Figure 2.11 (A, B, C), the master mould fabrication process seemed successful at producing accurate and repeatable patterns and demonstrated the ability to vary both the diameters of the holes as well as the distance separating them. Image D shows the cross section of the mould, demonstrating the ability to manufacture a cup like reservoir through RIE, and a through hole feature through DRIE.

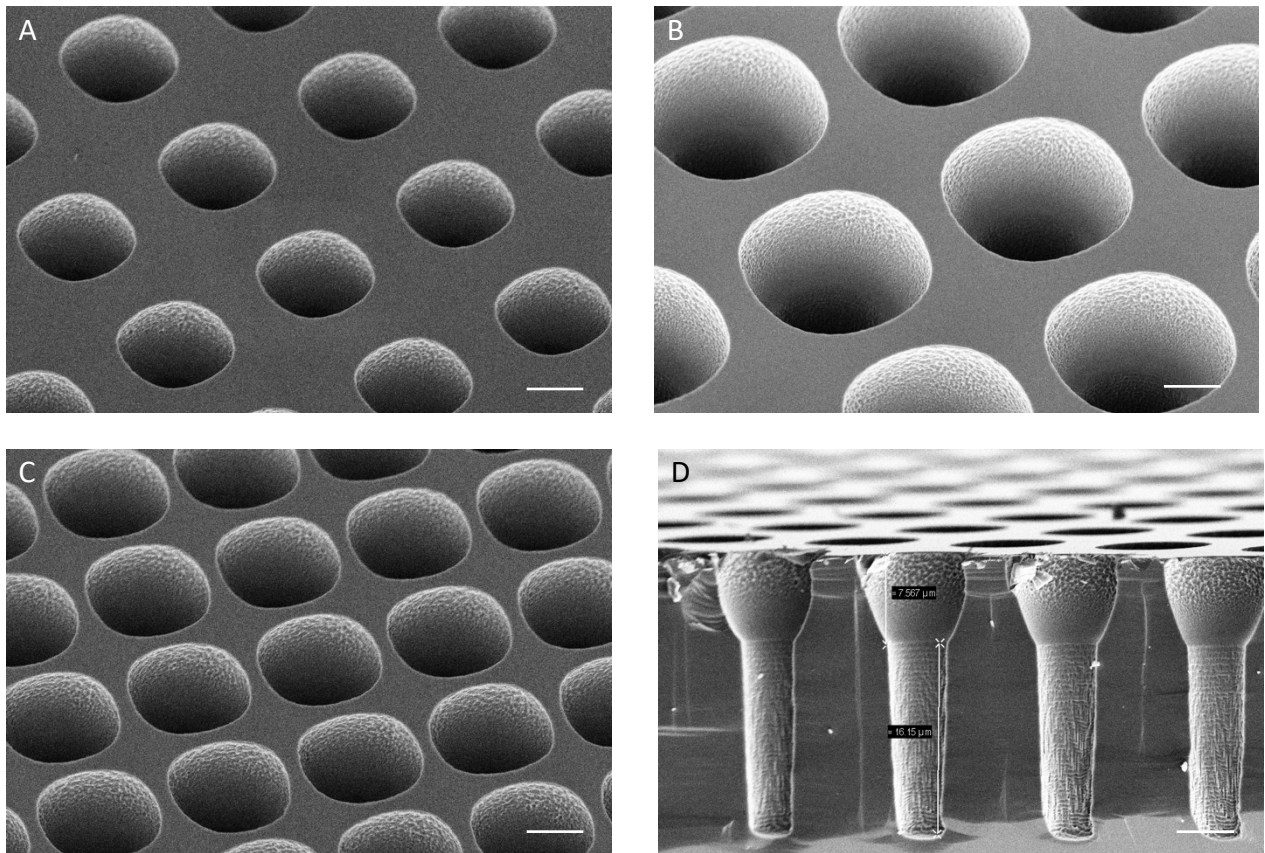


Figure 2.11: SEM images of representative conditions. SEM images of conditions 3 (A), 6 (B) and 4 (C) at 5000X magnification. (D) SEM image of the cross section of condition 3. All images were taken by Dr. Hyejeong Seong using the LEO Gemini 1525 FEGSEM (Zeiss). Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bars: (A), (B) and (C): 5 μm . (D): 4 μm .

2.2.2 PDMS negative mould fabrication

The fabrication step that followed was to produce the negative of this mould using polydimethylsiloxane (PDMS, Sigma-Aldrich). To prepare the surface of the wafer for the PDMS, and to make sure the PDMS mould can be demoulded from the silicon master mould, the wafer was coated in a thin layer of hexamethyldisilazane (HMDS, 97%, Sigma-Aldrich). This was accomplished by placing both the wafer and a 5 mL solution (in a small open container) of HMDS into a sealed container and heating them on a hot plate for an hour at 60°C, to allow the HMDS to evaporate and deposit onto the wafer, coating it in a thin layer and making the surface hydrophobic [171]. This process was repeated for each demoulding repeat.

The PDMS was prepared by mixing the silicone elastomer with the curing agent in a 10:1 ratio respectively and degassed for 1 hour in a desiccator to remove any bubbles that might be present in the PDMS solution. A thick layer (approximately 5 mm) of the PDMS solution was poured onto the silicon wafer and allowed to cure at room temperature overnight. The following day, the PDMS negative layer was carefully delaminated from the wafer and was ready to be used to create the positive hydrogel mould for the photoreceptor cultures, as seen in Figure 2.12. SEM imaging was conducted on all conditions to verify the accuracy of this method, and to visualise the negative columnar structures. The images are organised in Figure 2.13 below. The PDMS negative mould can be sterilised and reused several times without damaging the structures, making it a suitable material for this purpose.

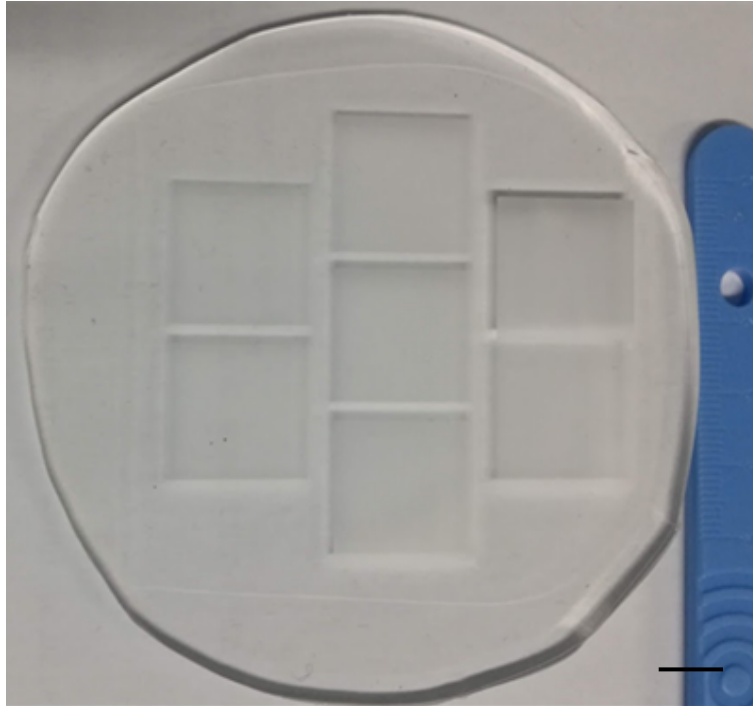


Figure 2.12: PDMS negative mould. The PDMS negative mould was fabricated from the master mould with the assistance of Dr. Hyejeong Seong. Scale bar 1 cm.

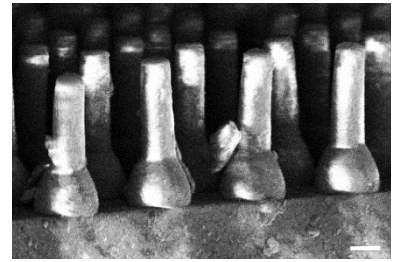
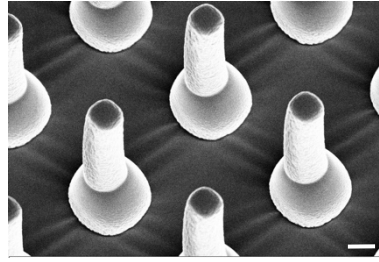
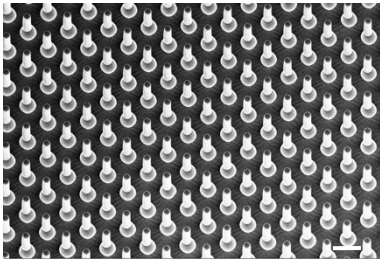
Figure 2.13: SEM images of all 7 conditions. Column 1 images are taken at a 45° angle at 1000X magnification, scale bar 20 μm . Column 2 images are taken at a 45° angle at 5000X magnification, scale bar 4 μm . Column 3 images are taken at a 90° angle at 4000X magnification, scale bar 5 μm . All images were taken by Dr. Hyejeong Seong using the LEO Gemini 1525 FEGSEM (Zeiss). Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. The figure can be seen on the next page.

1000X

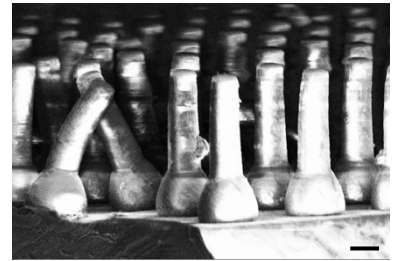
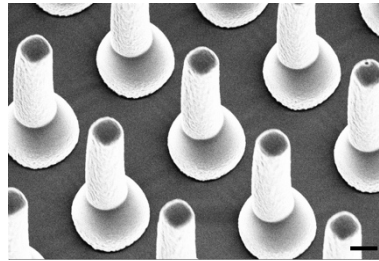
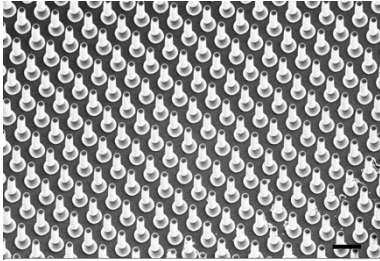
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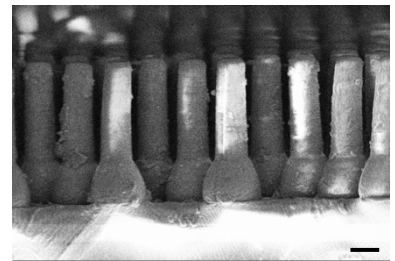
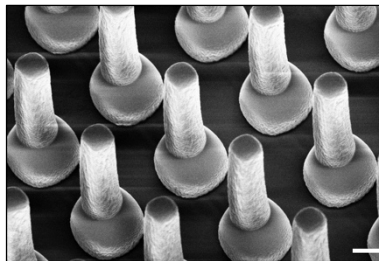
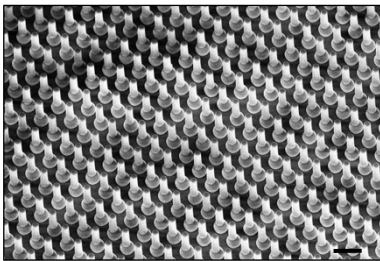
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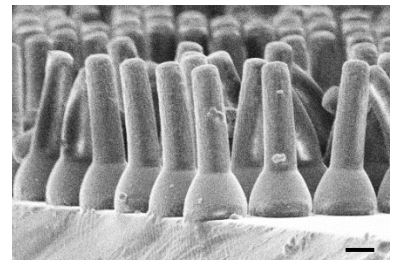
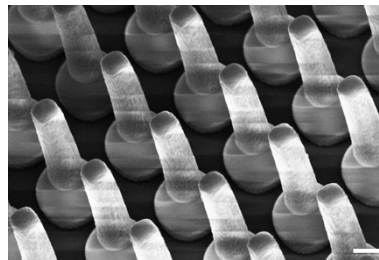
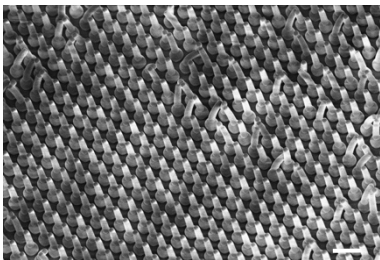
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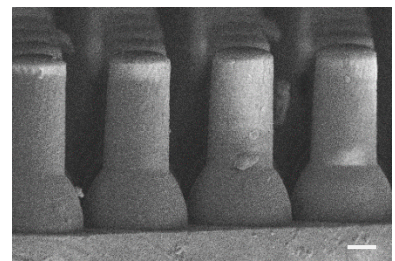
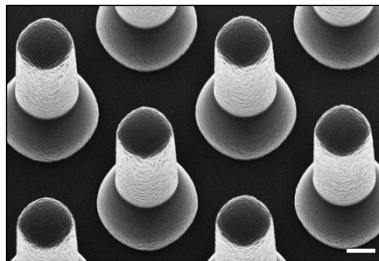
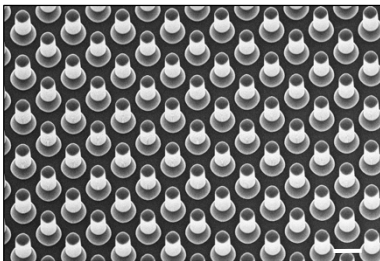
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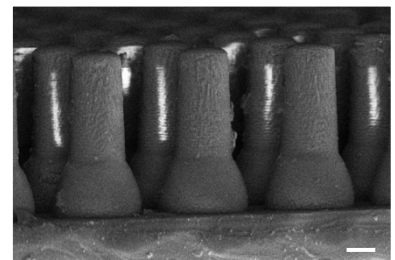
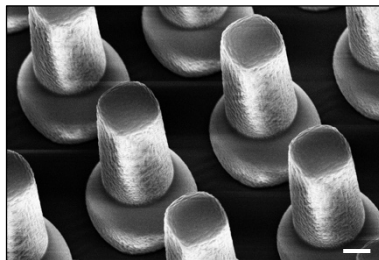
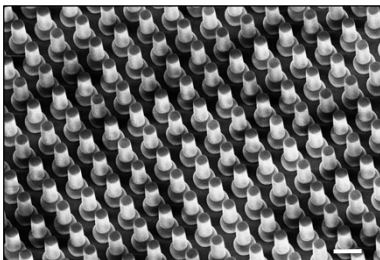
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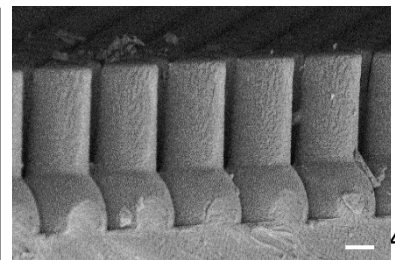
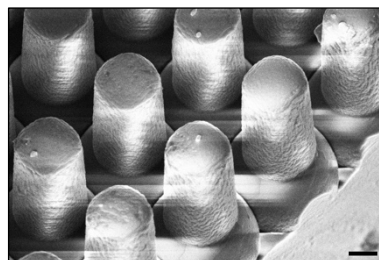
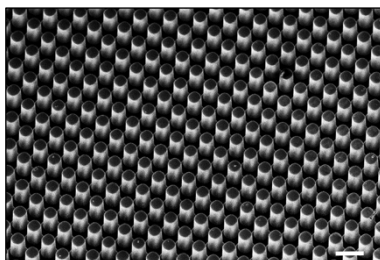
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2.2.3 Final scaffold fabrication

The final scaffold was fabricated by Dr. Eoghan Cunnane on the mould with dimensions $D = 5\ \mu\text{m}$ and $d = 15\ \mu\text{m}$ and using a solution of 55% functionalised GelMA at a concentration of 180 mg/mL.

The gel precursor solution was prepared by weighing the lyophilised GelMA in an Eppendorf tube and adding PBS to dissolve it. The solution was then placed in an oven at 60°C for an hour to dissolve the GelMA fully and to decrease the viscosity of the solution, thereby making it more handleable. Following this, the LAP was added at a concentration of 0.5 mg/mL, the tube was kept covered in aluminium foil to prevent any polymerisation from the surrounding light, and then vortexed to dissolve the LAP into the GelMA.

To fabricate the scaffold, 300 μL of the prepared solution was pipetted onto a clean unpatterned wafer surface, and the PDMS negative mould was added onto the solution with the columnar structures facing down into the solution. A clear glass 60 g weight was added on top of the mould to push it down onto the solution. The setup was placed under the UV lamp for 10 minutes to cure the gel. Once fully polymerised, the hydrogel, still stuck to the mould, was removed from the wafer, and then the hydrogel was carefully delaminated from the mould with fine tip forceps. The patterned hydrogel was left to dry before being imaged on a bright field microscope at 40X magnification. The gel was imaged from the top to the bottom at several heights to take representative images of the pattern throughout the hydrogel and can be seen in Figure 2.14.

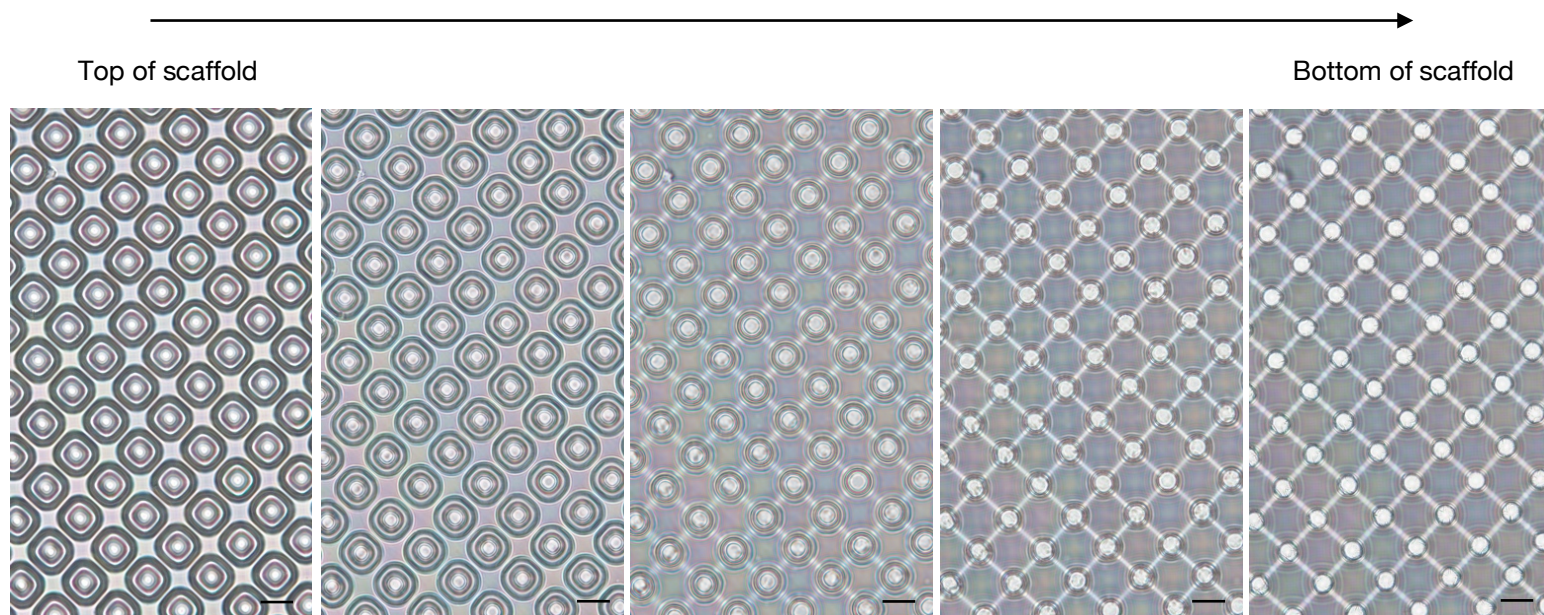


Figure 2.14: Images of patterned hydrogel. Patterned hydrogel imaged from top to bottom to visualise the pattern. Images were taken by Dr. Eoghan Cunnane. Scale bar 10 μm .

2.2.4 Limitations of this experiment

The first version of this hydrogel scaffold was based on the Jung *et al.* 2018 paper, however, it had a significant limitation. In their design and their initial cell culture experiments, they seeded their photoreceptor cells at a density of 8,900 cells per mm^2 [98]. From their scaffold design, it can be determined that a 1 mm^2 area of their scaffold contains approximately 3900 channels (Figure 2.15). Additionally, it can be calculated that each channel contains an average of 2.3 cells.

Theoretically, for spheres of equal dimensions, the densest packing possible is approximately 74% of the total volume of the space. However practically, when spheres are randomly dropped into a space, the actual packing density ends up being around 63% [172]. Applying these numbers to the current geometry, the cell body diameter can

be approximated to $5\text{ }\mu\text{m}$, making the volume of the cell $65.5\text{ }\mu\text{m}^3$. Assuming the reservoir can be estimated as a half sphere with diameter $15\text{ }\mu\text{m}$, the volume of the space is $883.6\text{ }\mu\text{m}^3$. With 63% packing, the number of cells that can fit into the reservoir space is calculated as 8.5 cells.

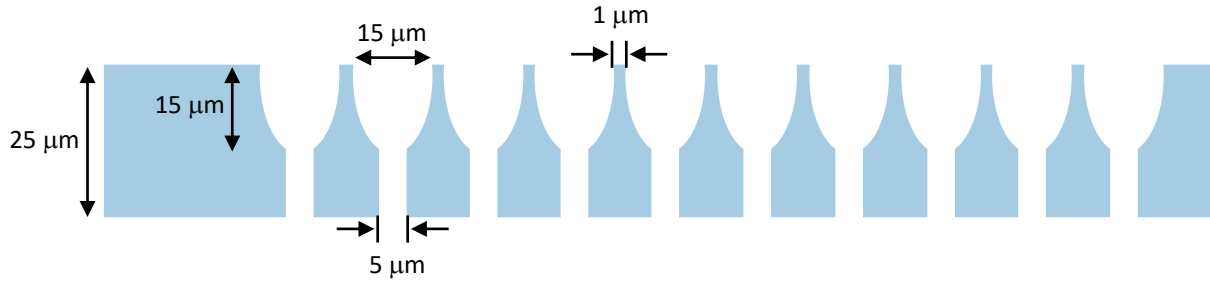


Figure 2.15: Schematic of the micropatterned hydrogel. Cross section illustration of the micropatterned hydrogel including the dimensions of the channels. Figure redrawn from [98].

Despite theoretical predictions, the authors were only able to fit approximately 2 to 3 cells per channel. Once stacked in the channel, these cells would be piled up in either one or two layers only. In comparison, the human retina, as well as the mouse one as seen in Figure 2.16, have a photoreceptor layer that is about 8 to 10 cells deep [173]. This property of the photoreceptor layer is essential for vision, and more specifically, high-resolution visual acuity in the macula. Studies have shown that the thickness of the photoreceptor outer nuclear layer is used to determine the health of the eye (Lujan 2015), with diseased eyes having a thinning layer thickness, due to loss of photoreceptors, leading to loss of vision [174]–[176]. The photoreceptor cell density in the native photoreceptor layer is about $150,000\text{ cells per mm}^2$ [177]. Therefore, this scaffold design is not able to achieve a density close to that of the native retina.

This low photoreceptor density was also highlighted as a major limitation by the collaborators at KCL, and one that needed to be adjusted in order to have successful post transplantation results *in vivo*.

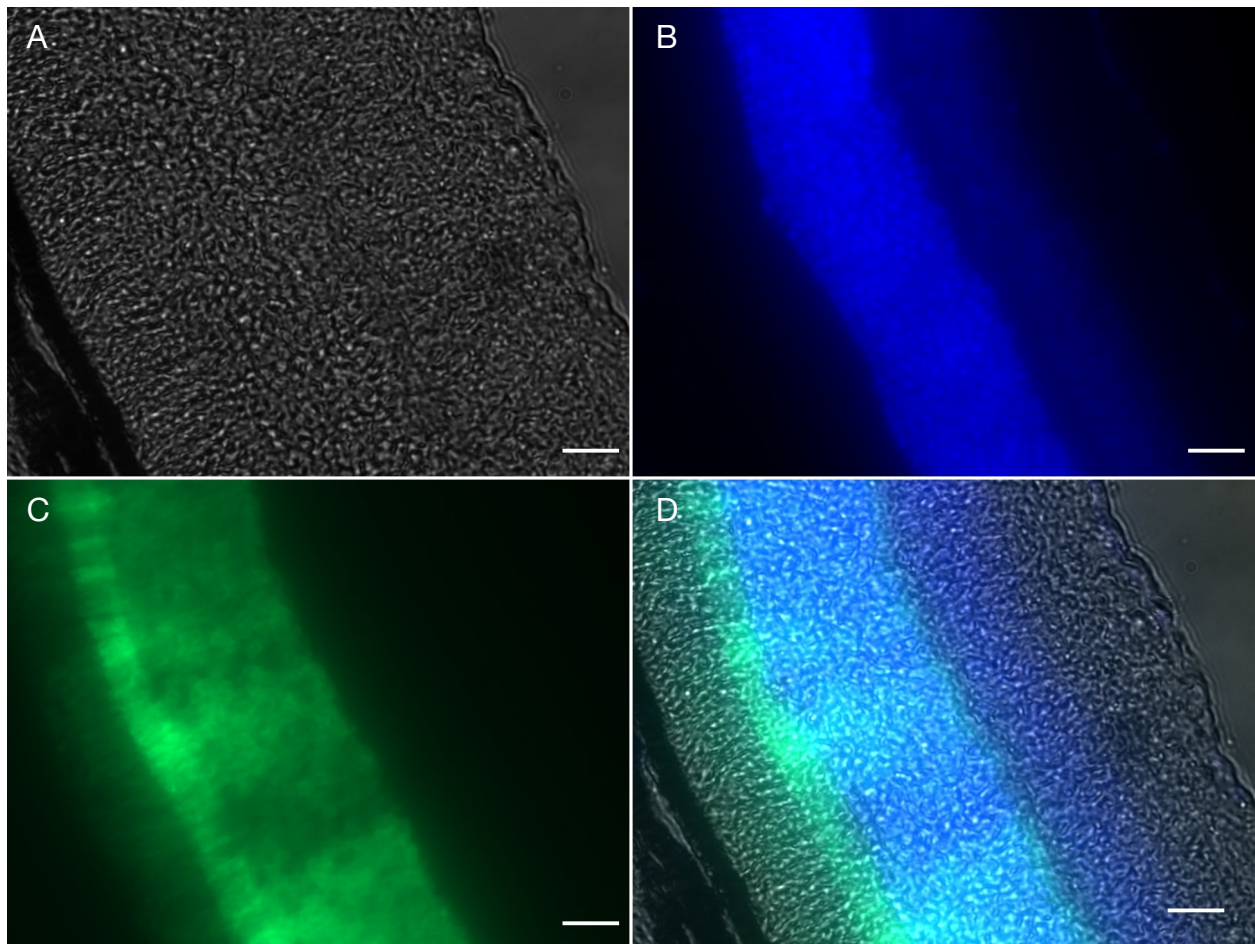


Figure 2.16: Microscopy images of a mouse retina. Images were taken using a Zeiss Axio Observer 1 microscope. The nuclei were stained with DAPI (blue), and the photoreceptors were green due to the GFP mouse model. Images with taken with the assistance of Dr. Joana Ribeiro, a previous student in the Ali group. The images are presented in the following channel order: widefield (A), DAPI (B), GFP (C) and their composite image (D). Scale bar 20 μm .

2.2.5 Choice of biomaterial

Even though GelMA did show promising results, recent literature has highlighted that HAMA plays a positive role in maintaining photoreceptors viability *in vitro* and *in vivo*. These publications were discussed in Chapter 1 but will be briefly mentioned again to justify changing the biomaterial from this point forward in the thesis.

Liu *et al.*' 2013 paper reported that following transplantation of hyaluronic acid-based hydrogels, encapsulating retinal progenitor cells, into the mouse retina, the cells were able to integrate into the host retina, differentiate, and expressed recoverin, a marker for mature photoreceptors [89]. Ballios *et al.*'s 2015 paper (following their 2010 paper) demonstrated that using a hyaluronan-based hydrogel improved the survival and integration of transplanted retinal stem cell-derived rods when compared to simple cell injections in a saline solution. They attributed these results to the interactions of a cell adhesion receptor (CD44) with the hyaluronic acid [40], [42]. Mitrousis *et al.*'s 2016 paper also established that hyaluronic acid-based hydrogels increased photoreceptor survival and maturation *in vitro* significantly [90].

Thus, the material used to fabricate these patterned scaffolds was changed to HAMA and will be explored further throughout the rest of this thesis.

2.3 Scaffold design 2: 'Champagne flute' channels scaffold

To overcome the cell seeding density limitation of the 'wine glass' channel geometry, the scaffold model was modified into a 'champagne flute' channel geometry, as illustrated in Figure 2.17. In this modified design, each channel would theoretically be able to contain a higher number of photoreceptor cells, up to 30 cells per channel, making the seeded photoreceptor layer closer in thickness, and in total number of cell layers, to the native retina. With this method, the theoretical number of cells that can be seeded and cultured increases to approximately 75,000 cells per mm², which is still half the number

of cells compared to the 150,000 cells per mm^2 of the native retina [177], but almost an 8.5-fold increase from the 8,900 cells per mm^2 that the previous design could hold. Also, the total thickness of the scaffold would be approximately 100 μm , closer to the actual thickness of the photoreceptor layer, which is between 90 and 100 μm [178]. The initial geometry design had a full height of 100 μm , which included a 10 μm stem at a diameter of 3 μm , and a larger chamber or reservoir at a diameter of 15 μm .

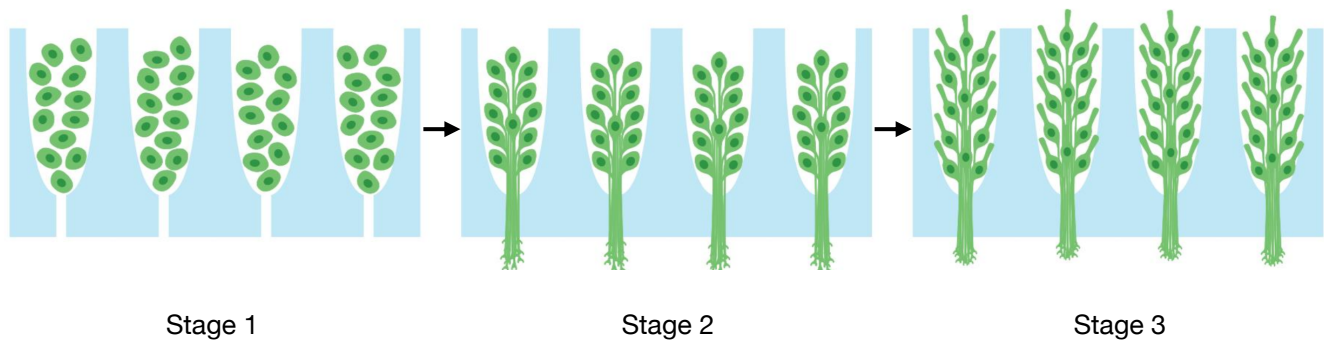


Figure 2.17: 'Champagne flute channels scaffold. The scaffold contains photoreceptor cells with morphological predictions about their different stages of culture. Stage 1: Photoreceptor cell bodies lose their outer segments and axons following sorting. Stage 2: Photoreceptor cells begin forming axons that grow into the stems of the scaffold. Stage 3: Photoreceptor cells being growing outer segments.

2.3.1 Possible fabrication approaches

There were several possible fabrication methods to produce these scaffolds, as illustrated in Figure 2.18. These methods were discussed, and their advantages and disadvantages were weighed before the final selection was made.

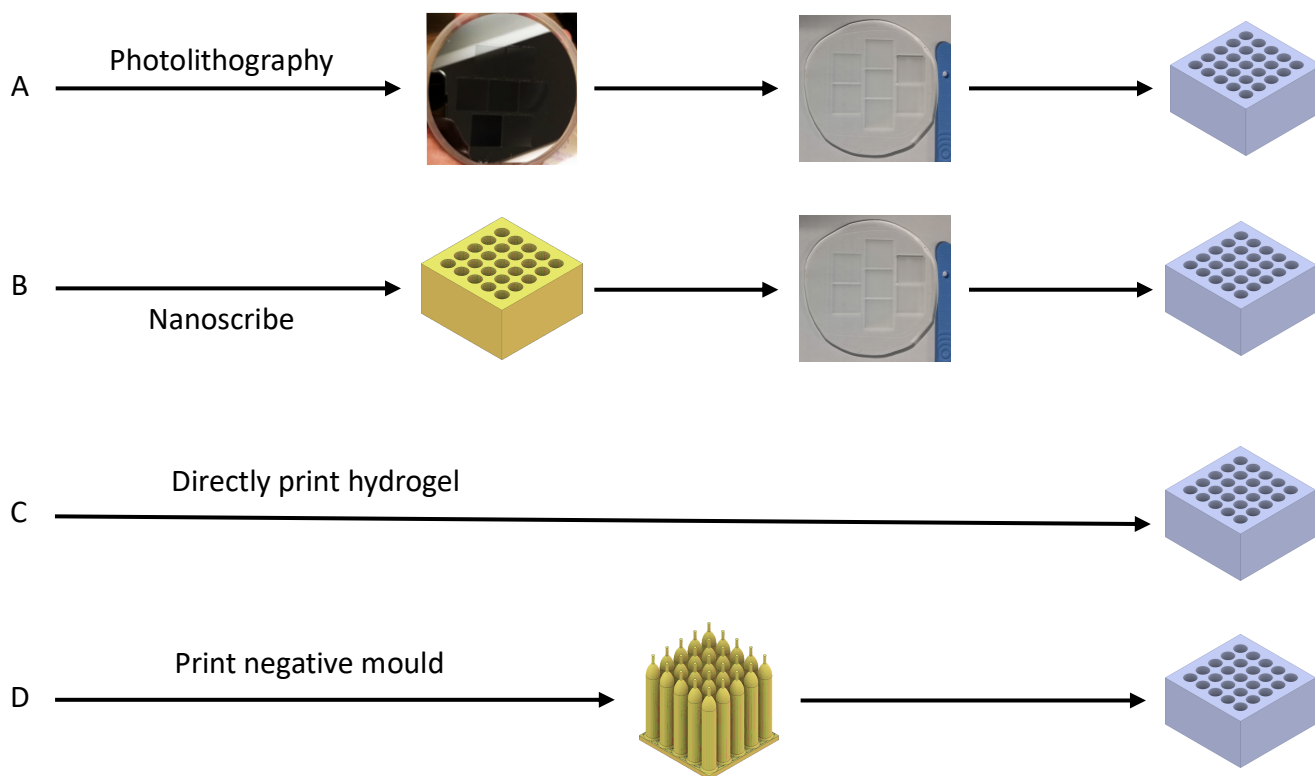


Figure 2.18: Possible fabrication approaches to produce the patterned hydrogel scaffold. Structured coloured in yellow are the 3D printed resin moulds, and the ones coloured in blue are the hydrogel scaffolds.

The first method (A) is the same one used to fabricate the ‘wine glass’ channel geometry: photolithography. Even though this method is commonly used and yielded successful results with the first iteration of the design, the modified design positive master mould could not be fabricated with this method. This was due to the significant increase in the total height of the channels, from $25\ \mu\text{m}$ to $100\ \mu\text{m}$, and the limitation of the RIE method depth that can be achieved with isotropic etching. During isotropic etching, material is removed in all directions under the photomask at the same rate, while during anisotropic etching, it is mostly removed in the vertical direction, as seen in Figure 2.19 [179]. As the isotropic etching method is needed to create the curvature of the reservoirs in the master

mould, present at the bottom of the channels, it is not possible to etch the modified geometry using this technique.

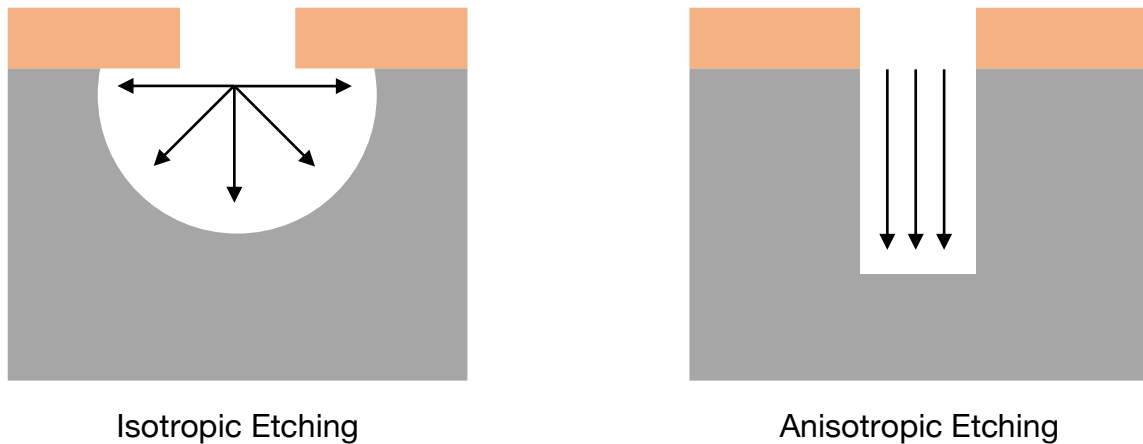


Figure 2.19: Schematic comparing the etching geometry for isotropic and anisotropic etching. During isotropic etching, material is removed in all directions under the photomask at the same rate, while during anisotropic etching, it is mostly removed in the vertical direction.

The second method (B) consisted of fabricating the positive master mould using 3D printing techniques. As the channels are microchannels, with the smallest feature being the stems at $3\text{ }\mu\text{m}$ in diameter, traditional 3D printers are not capable of printing this mould. Some examples of these printers and their resolutions include selective laser sintering ($100\text{ }\mu\text{m}$), stereolithography ($5\text{ }\mu\text{m}$), digital light processing ($5\text{ }\mu\text{m}$), inkjet ($10\text{ }\mu\text{m}$) and fused deposition modelling ($100\text{ }\mu\text{m}$). Only two photon polymerisation (TPP) printers can print such structures with a resolution of 100 nm [180]. The only TPP printer commercially available that can print this structure is the Nanoscribe printer (Nanoscribe GmbH). Even though it is possible to print the structure successfully, the limitation of this technique is in the second stage of fabrication: the PDMS negative mould fabrication step. The negative mould would contain columnar structures of height $100\text{ }\mu\text{m}$ and

diameter 15 μm , making their aspect ratio 6.67:1. Although the literature has shown that PDMS can be used to create high aspect ratio structures [181], it was also stated that these PDMS structures will tend to stick to each other when they are in close proximity, with the critical aspect ratio value (that leads to failure) decreasing as the spacing decreases. This failure mode is called lateral collapse, and is due to the surface adhesion of PDMS, as well as its elastic modulus [181]. Due to the minimal spacing between the features in this mould, and the higher risk of the structures sticking to each other, this method was not chosen.

The third method (C) has been previously published and described in Chapter 1 [101], [102]. It consisted of using the Nanoscribe to directly print the hydrogel scaffold using a photocrosslinkable polymer. However, it was decided that this method required extensive optimisation [100]–[102], which would not be possible given the limited amount of time allocated to this project on the Nanoscribe available in the group. Furthermore, this method could not be used for high throughput production as each print would be used only once, unlike the previous methods that produce a reusable negative PDMS mould.

The fourth method (D) that was considered, and ultimately chosen, was to use the Nanoscribe printer to print the negative columnar structures. Unlike the second method, these structures would be more rigid, with a Young's modulus of 5.11 GPa, compared to the PDMS's ranges from 360 to 870 kPa [182], and able to preserve their shape for the demoulding step with the hydrogel. Furthermore, the negative mould would be reusable thus making it a higher through put fabrication technique.

2.3.2 Nanoscribe printer

The Nanoscribe printer was purchased and new to the group in summer of 2019, meaning there was no expertise on how to use it efficiently, therefore it was a learning process for members of the group. The Nanoscribe is a 3D printer that uses TPP to

create three-dimensional micro and nano-structures. This process is based on direct laser writing (DLW) but works instead with a two-photon absorption (TPA) setup. TPA is a non-linear absorption process during which two photons are absorbed simultaneously by a molecule, allowing an electron to be promoted from a lower energy level to a higher one [183].

To print the structure, the 3D model was created on a CAD software (Autodesk Inventor) and saved as an STL file. There are two Nanoscribe softwares that are provided by the company. The first Nanoscribe software is called DeScribe, and it is the software into which the STL file is imported. This software produces a GWL file that can be read by the second Nanoscribe software called NanoWrite, which is setup to the printer and starts the printing.

DeScribe reads the STL file and goes through a series of steps to create the print path for the 3D model. Initially, the model is sliced into horizontal planes called slices. The slicing distance depends on the solution set being used. Then, each slice is divided further, or hatched, and the print path is defined for each slice. Finally, the software allows the user to either print the structure directly, or to create a defined array of this structure and print that. If the print is larger than the print field diameter (which is defined by the objective used), the software also divides it up into blocks and stitches them together by slightly overlapping the prints to allow for better adhesion of the blocks and to smoothen the connections between the blocks. The laser power and the scanning speed can be defined in the last step of the program.

Depending on the dimensions of the model and the resolution required to print it, a solution set is chosen. With the current printer setup in the group, there is the option of choosing one of three solution sets: one for small features (3D SF) using a 63x objective, one for medium features (3D MF) using a 25x objective, and one for large features (3D LF) using a 10x objective. Each of these solution sets is a combination of substrate and resin, as well as a printing configuration. For this model feature dimensions, the 3D MF

solution set was chosen, which is the set for 3D microfabrication of medium features (Figure 2.20).

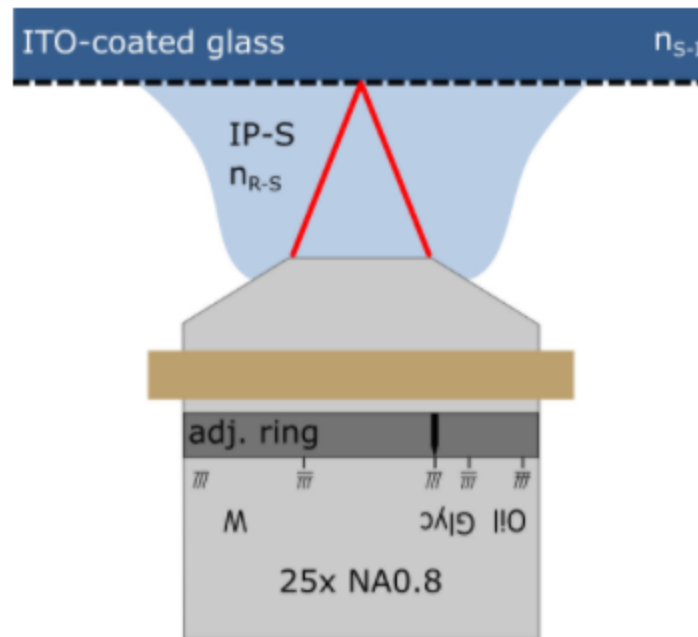


Figure 2.20: 3D medium features solution set. Figure reproduced with permission from [184].

This set requires using the 25x objective with the ITO-coated glass substrates as well as the IP-S resin. The objective also defines the print field diameter, which is 400 μm in this case. There are also two recipe options for prints, either solid or shell (solid prints for this project models), with a minimum hatching distance of 0.5 μm and a minimum slicing distance of 1 μm . The ITO substrate is made up of two components: the soda lime glass with a refractive index of 1.518 @ 780nm, as well as the ITO coating (iodium-tin oxide) with a refractive index of 1.624 @ 780nm with a film thickness of 18 nm $\pm$ 5 nm. The refractive index is relevant because it is how the machine finds the interface of the

substrate to be able to print directly on its surface. The IP-S resin formulation is proprietary.

After the print is completed, the substrate with the print attached to it is submerged into a solution of propylene glycol monomethyl ether acetate (PGMEA, Sigma-Aldrich) for 20 minutes, followed by a solution of isopropyl alcohol (IPA, Sigma-Aldrich) for 5 minutes, to remove the uncrosslinked resin. The substrate is then left to dry fully and placed in a curing chamber for 15 minutes to ensure that the print is fully cured. This final step is not as important for solid prints, as the print should be fully cured, however it is a crucial step when printing a shell structure, during which the printer only prints the outside shell of the model, and internal trusses to support the print. The rest of the interior of the print is uncured resin that needs to be post cured.

2.3.3 Printing optimisation

The first step in printing the negative mould was to draw up a singular column, with the dimensions seen in Figure 2.21 and print an array of 50 x 50 singular columns to form a 1 mm² square containing a total of 2500 columns with the features spaced at 20 µm from each other. When the software prints an array, it prints each feature fully before moving on to the next one and stitches the overlapping sections together to allow for better adhesion of the full print. A 5 µm thick base, with an area of 400 µm² (20 µm x 20 µm), was added to the column to increase the contact area of the print with the substrate, and to create a larger area of overlapping for the individual prints of the array. This final structure can be seen in Figure 2.22 (A).

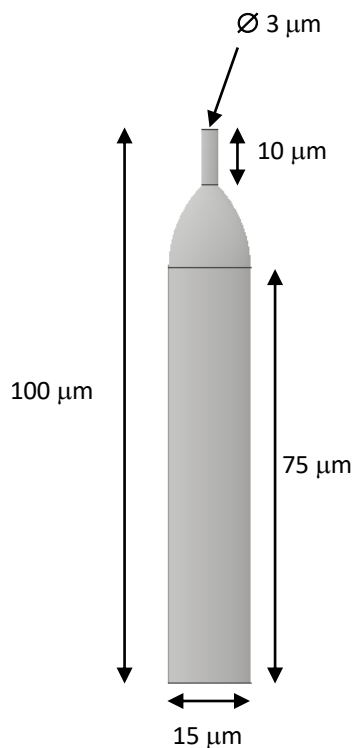


Figure 2.21: Dimensions of single 3D column, modelled on Autodesk Inventor.

Once the print was completed, it was quickly imaged on a light microscope to verify the success of the print (Figure 2.22 (B)). It was then cleaned using the described protocol and imaged again to check the structures (Figure 2.22 (C)). Unexpectedly, the print was damaged during the cleaning process and the structures seemed to detach from the substrate or bend towards neighbouring ones. This print was repeated 6 times, and the resulting structures were consistently damaged after the clean. Images were taken of one of those repeats after the washes, using a light microscope (Figure 2.22 (D)) and with the SEM (Figure 2.22 (E)).

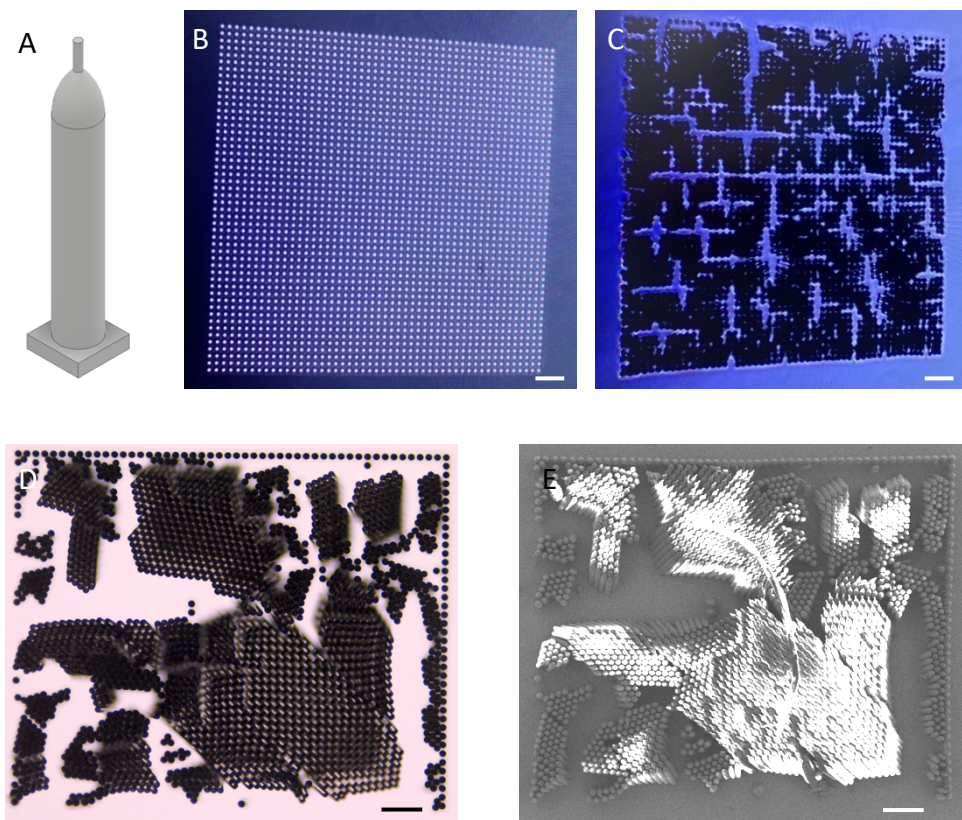


Figure 2.22: Images of the initial printing attempts. (A): Single 3D column structure. (B), (C) and (D): Light microscope image taken using an EVOS XL core microscope (Thermofisher). (E): SEM image at 150X magnification taken using the LEO Gemini 1525 FEGSEM (Zeiss). Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bar 100 μm .

To overcome the issues caused by the cleaning steps, the 3D model was modified, and instead of a singular column, it was changed into an array of 10 x 10 columns on a continuous 5 μm thick base with a total area of 0.04 mm^2 (Figure 2.23 (A)). This modification resulted in a successful print and cleaning process, and this was validated with light microscopy (Figure 2.23 (B)) and SEM (Figure 2.23 (C)). The reason behind the success of this approach compared to the last one is because the printer software slices the entire 3D model into planes and prints it in that way as well. Thus, the base is printed

as a whole, and is better attached to the substrate, and the features are adhered sufficiently not to be damaged by the cleaning process.

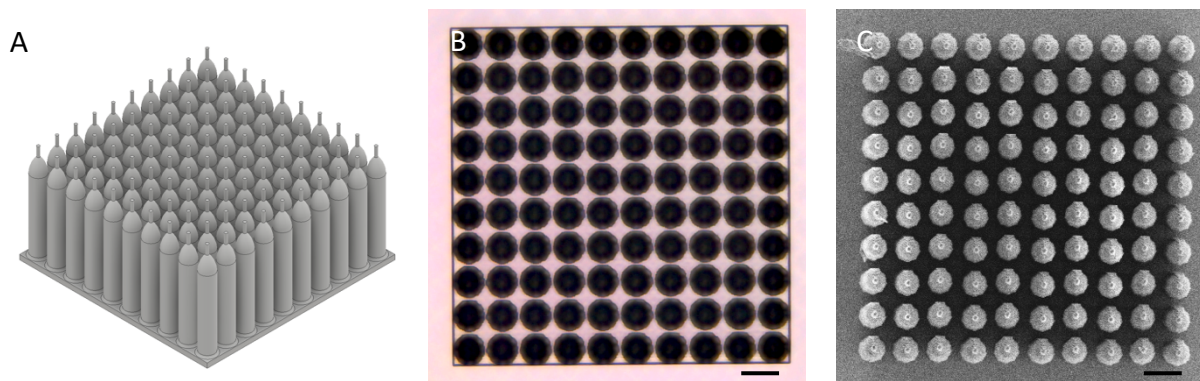


Figure 2.23: Images of the 10 x 10 array. (A): 3D array of 10 x 10 columns. (B): Light microscope image taken using an EVOS XL core microscope (Thermofisher). (C): SEM image at 500X magnification taken using the LEO Gemini 1525 FEGSEM (Zeiss). Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bar 20 μm .

The next step was to print a 1 mm² square using the same 3D model. An array of 5 x 5 repeats of the 3D model from Figure 2.23 (A) was drawn up on Describe (Figure 2.24 (A)) and printed (Figure 2.24 (B)). In a similar way to the previous attempt, the printer prints each individual 0.04 mm² model first, and then moves on to the next one. This print took approximately 45 minutes to complete and yielded good results with no sections of the print peeling off during the cleaning process.

It must be noted that the areas where the blocks are stitched can be visualised once the print is cleaned. It must also be mentioned that there are slight deformations visible in the print (Figure 2.24 (B)), which is due to issues with the printing power. This will be discussed and addressed later in this chapter.

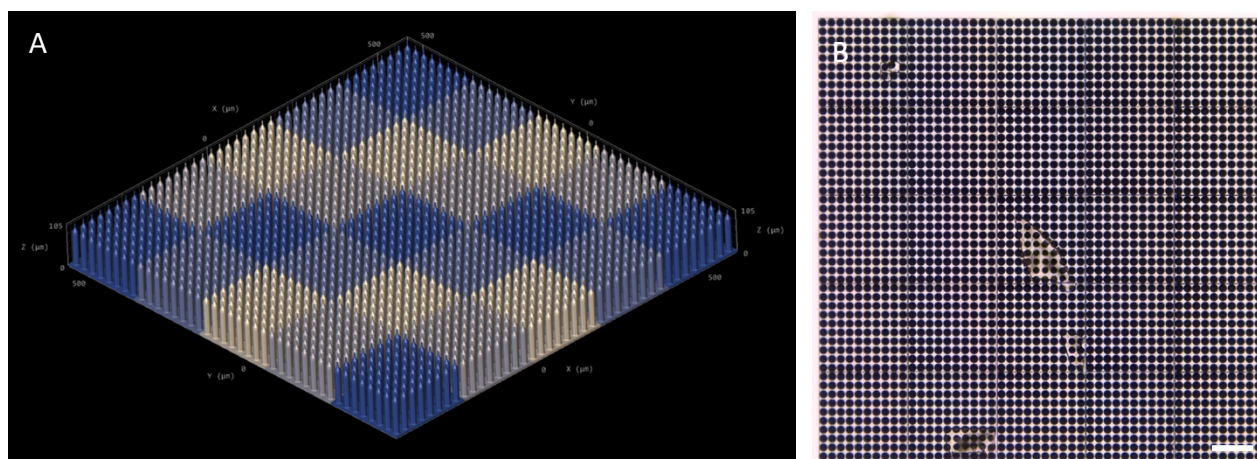


Figure 2.24: Images of the 1 mm² print. (A) 3D array of 10 x 10 columns. (B): Light microscope image of the cleaned print taken using an EVOS XL core microscope (Thermofisher). Scale bar 100 μm.

In order to demonstrate the scalability of this printing technique, a 5 mm x 5 mm print, which includes a 25 x 25 array of the model from Figure 2.24, was printed successfully (Figure 2.25). However, with increasing print area, there is a significant increase in printing time. A 1 mm² print takes approximately 45 minutes to print, while a 25 mm² print takes 25 times as long, making it almost a 19-hour print. Therefore, for the purpose of optimisation, it was decided to continue printing structures approximately 1 mm².

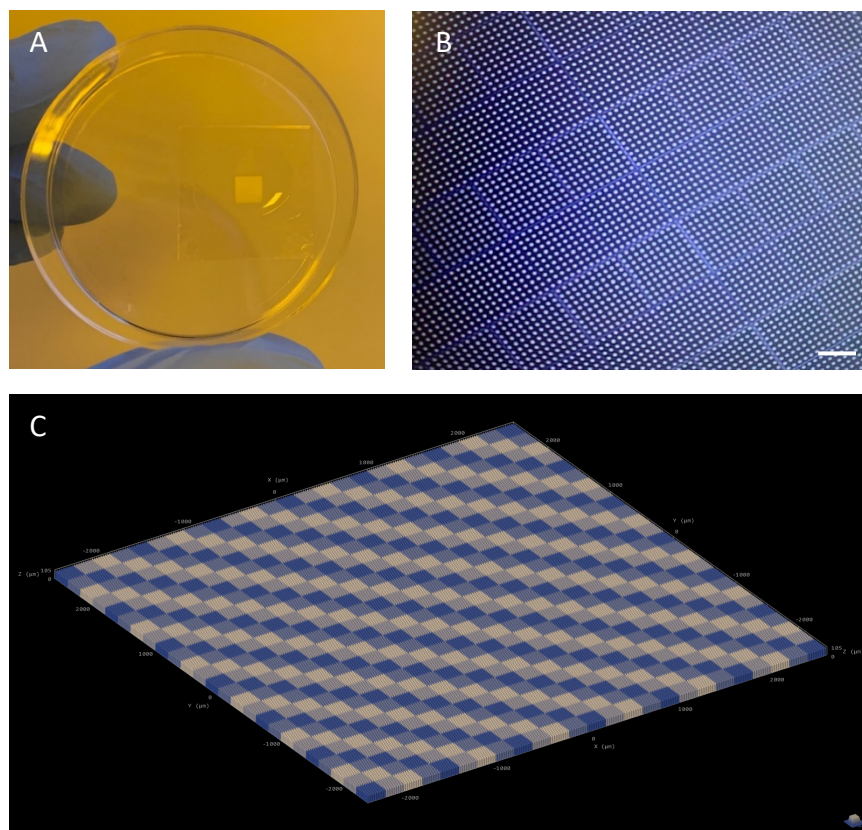


Figure 2.25: Images of the 5 mm² print. (A): Printed array of 25 x 25 models. (B): Light microscope image of the cleaned print taken using and EVOS XL core microscope (Thermofisher). (C) 3D array of 25 x 25 models. Scale bar 100 μm.

2.3.4 Initial demoulding attempts

Following the successful printing of the various 3D models, the next step was to attempt to demould the positive patterned layer from the 1 mm² rectangular 20 μm spacing negative print. Initial demoulding tests were run with PDMS as it is an inexpensive and readily available material. The print on the substrate was plasma treated to clean the surface, and then silane treated to make the surface hydrophobic. The silane treatment was done using trichlorosilane (trichloro(1H,1H,2H,2H-perfluorooctyl) silane, Sigma-Aldrich). Briefly, the substrate was placed in a desiccator along with a small open vessel

containing 5 mL of the trichlorosilane solution. The desiccator was placed under vacuum and left for 1 hour for the solution to evaporate and deposit onto the substrate, coating it in a thin layer and making the surface hydrophobic. Following that, the substrate was transferred to an oven and left to bake at 60°C for 2 hours to cure the silane [185].

Contact angle images were taken by Dr. Eoghan Cunnane of the substrate untreated, after plasma treatment and after silane treatment, to visualise how the different treatments alter the hydrophobicity of the surface. The substrate was placed on a flat surface, and a drop of distilled water was added to it. The drop was imaged, and the contact angle was measured. The contact angle, θ , is a quantitative measurement of the wettability of a solid by a liquid. It is defined as the angle that is formed at the intersection of a liquid, solid and gas [186], [187]. This method of measuring this angle is the static method, also called the sessile drop. For each condition, four samples were used, and the values of θ were determined by drawing the tangent to the drop as illustrated in Figure 2.26. Representative images of the three different droplets as well as the averages and standard deviations of the measurements can be seen in Figure 2.27. The calculated values determine that the untreated substrate has a more hydrophilic surface with a contact angle $\theta < 90^\circ$, the plasma treated substrate has a super hydrophilic surface with a contact angle $\theta \cong 0^\circ$, and the silane treated substrate has a hydrophobic surface with a contact angle $\theta > 90^\circ$.

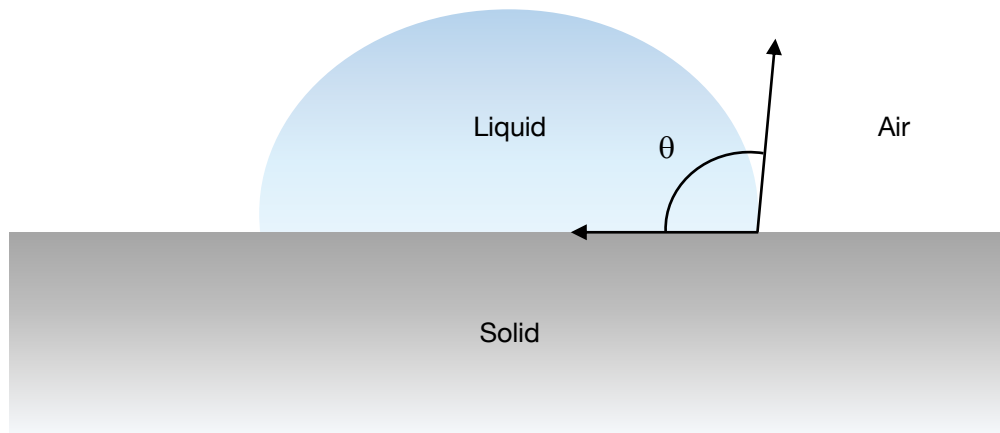


Figure 2.26: Schematic of a sessile drop contact angle system. The contact angle is defined as the angle that is formed at the intersection of a liquid, solid and gas. Figure redrawn from [186].

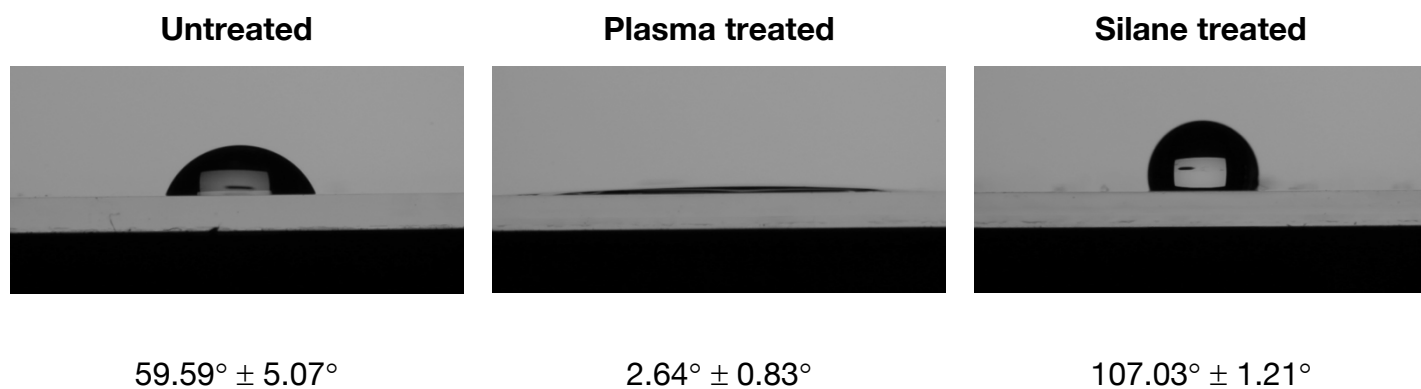


Figure 2.27: Contact angle images on different surface treatments. Images of droplet on untreated, plasma treated, and silane treated substrates, as well as the averages and standard deviations of the measurements for each condition. The data was generated by Dr. Eoghan Cunnane (n=3).

Following the silane treatment, the substrate surface was hydrophobic and ready for the PDMS to be moulded onto it. The negative print remained adhered to the glass substrate, and a 100 μm thick sheet of plastic (to match the height of the printed mould) was taped around the print to create a reservoir (Figure 2.28 (A)). Following that, a PDMS solution, prepared in the same way as described previously in section 2.2.3, was poured in excess onto the printed negative (Figure 2.28 (B)). The solution on the substrate was placed in the oven at 60°C for two hours to cure and solidify. Finally, the PDMS sheet was manually demoulded from the print and came off successfully without damaging the print (Figure 2.28 (C)).

The PDMS positive mould was imaged on the brightfield microscope (Figure 2.28 (D)) and on the SEM (Figure 2.28 (E) and (F)) to visualise the channels. The brightfield image showed the uniformity of the pattern and the successful transfer of the pattern, however it did highlight some irregularities at the interface between the stitched blocks, where some of the channels merged. This was due to the small spacing between the columns in the negative print, limiting the amount of material that could fit into the mould in those areas. This space was even more limited in the overlapping stitched areas of the blocks. The SEM images also showed that the pattern includes the stems visible at the bottom of the channels.

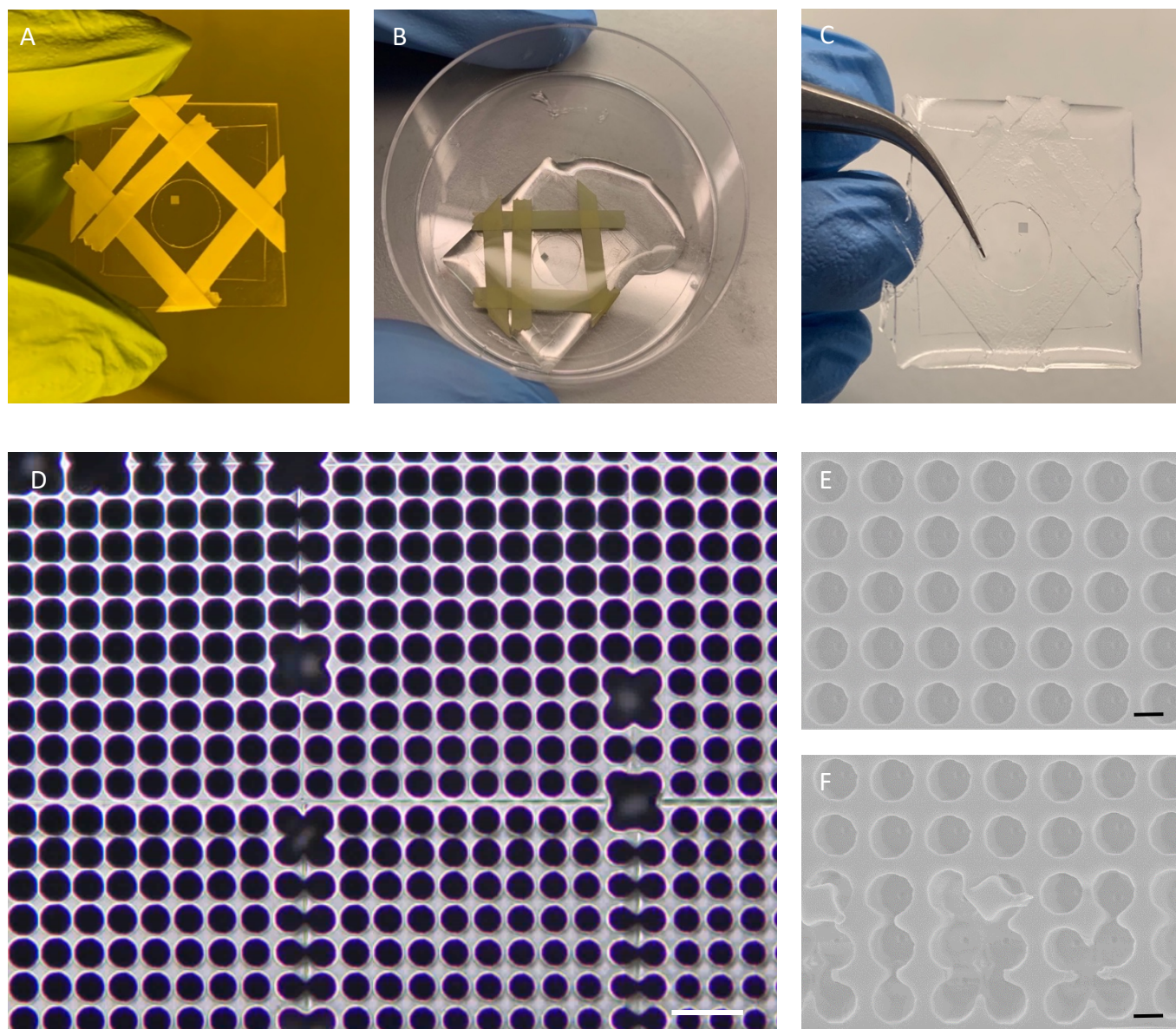


Figure 2.28: Images of the first PDMS demoulding attempt. (A): Picture of the substrate setup for PDMS demoulding. (B): PDMS layer poured onto the substrate. (C): Demoulded PDMS sheet including the transferred pattern. (D): Light microscope images taken before (B) and after (C) the cleaning step, using (). (E) and (F): SEM image at 2000X magnification, taken by Kun Zhou using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bars: (D): 50 μm , (E): 10 μm .

Since the demoulding was successful with this negative print, the next test conducted was to reuse that print but only add a thin layer of PDMS on top, as thin as the height of the printed columnar features, in order to get through-holes. Since the surrounding plastic sheet had a height of 100 μm , equal to the height of the print, the easiest approach was to use spin coating. A smaller amount of PDMS was poured onto the mould and the substrate with the PDMS was placed on the spin coater in order to get rid of the excess PDMS, thereby leaving solution in the reservoir created by the plastic sheet around the print. The solution on the substrate was placed in the oven for two hours to cure and solidify. The PDMS sheet was manually demoulded from the print (Figure 2.29 (A)), however, it tore as soon as it reached the edges of the printed negative (Figure 2.29 (B)). Tearing was attributed to the absence of excess PDMS to support the delamination.

These 2 experiments were only a proof of concept but did show promising results. Further modifications to the mould and demoulding approach were required to achieve successful scaffold fabrication.

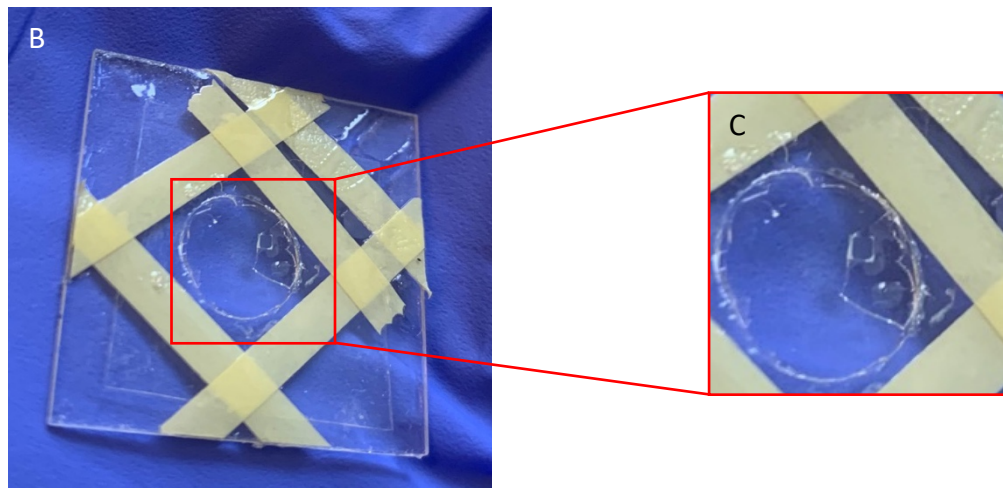
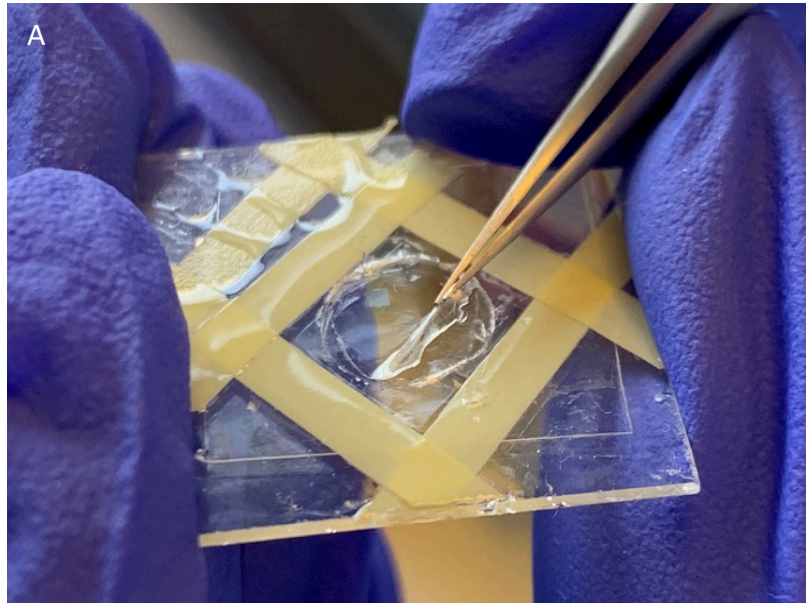


Figure 2.29: Images of the second PDMS demoulding attempt. (A): Picture of the PDMS being delaminated from the substrate. (B-C): Picture of the substrate with the torn PDMS around the edge of the print.

2.4 Modified 3D models and demoulding techniques

2.4.1 Varying the array spacing and geometry

As mentioned in the previous section, the 20 μm spaced columns in the negative print had limitations in terms of printing and demoulding, which consisted of irregularities in the overlapping stitched blocks of the print and merging of channels in the demoulded hydrogel due to the limited space between the columns. Therefore, two other models were drawn up with larger spacings, 25 μm and 30 μm , to make sure the channels were more clearly separate from each other. This would allow for enough material to fit into the mould, as well as avoid any irregularities on the stitched blocks. However, it must be noted that increasing the spacing also meant decreasing the number of channels and eventually the density of the cells in the hydrogel. The number of features in the different array spacings was calculated and can be seen in Table 2.1.

The details of the new models as well as the 20 μm spacing model can be seen in Figure 2.30 below for comparative purposes. These three different spacing variations were printed, imaged pre- and post- cleaning, and with the SEM. SEM images were taken by a PhD student in the group, Kun Zhou.

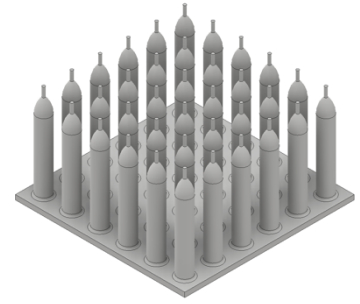
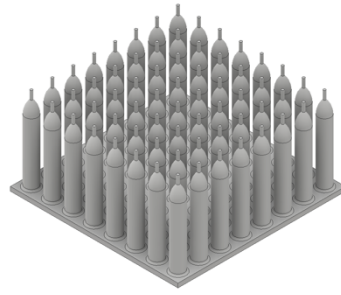
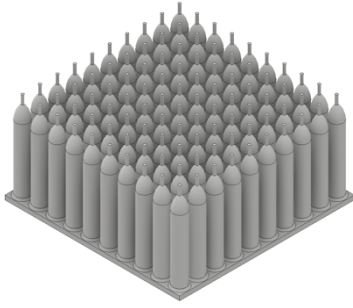
Figure 2.30: Images of the negative prints with three different spacings. (A): 3D array of columns. (B) and (C): Light microscope images taken before (B) and after (C) the cleaning step, using an EVOS XL core microscope (Thermofisher). (D), (E) and (F): SEM image at 200X (D), 1000X at 30° tilt (E), and 2000X (F) magnification, taken by Kun Zhou using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bars: (B), (C) and (D): 100 μm , (E) and (F): 10 μm . The figure can be seen on the next page.

20 μm

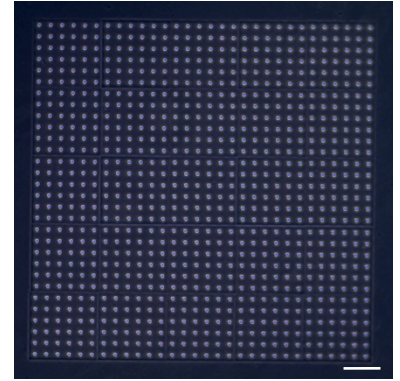
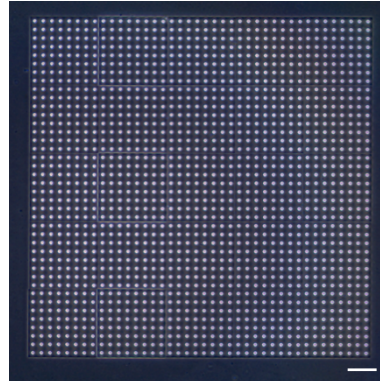
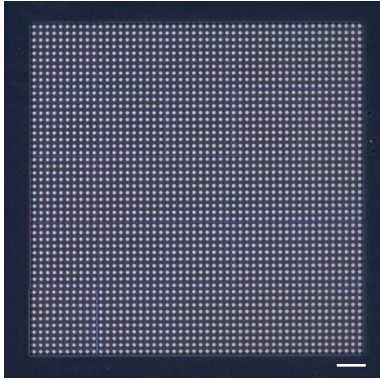
25 μm

30 μm

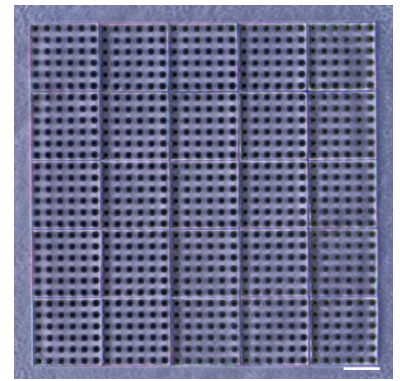
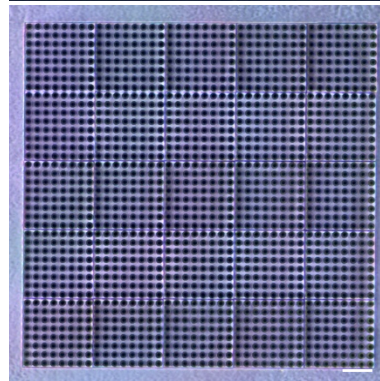
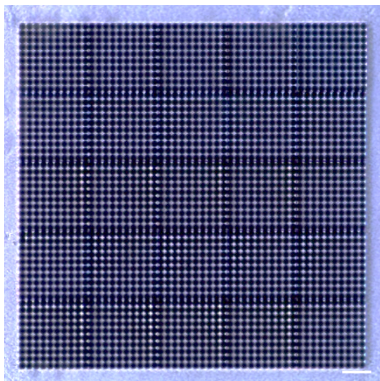
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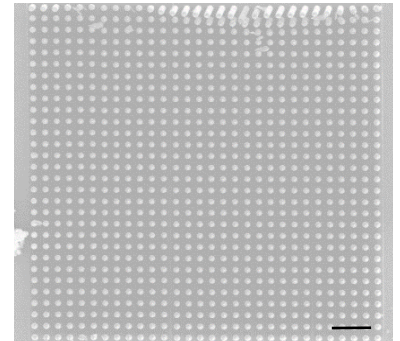
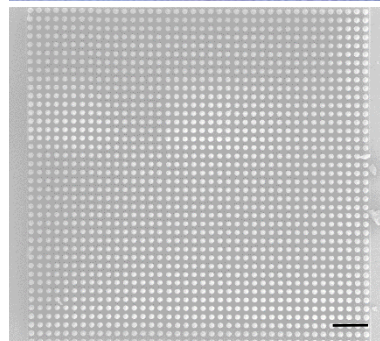
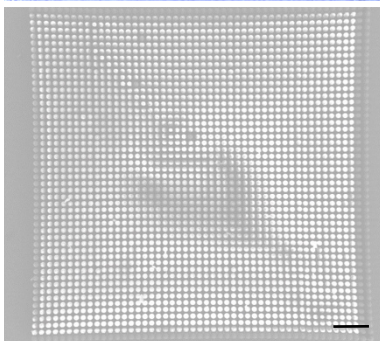
B



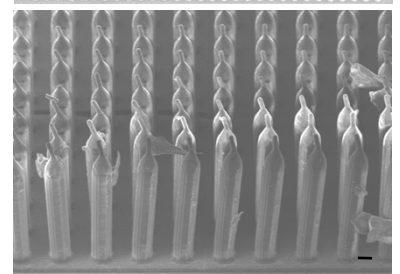
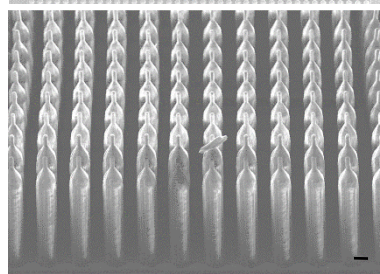
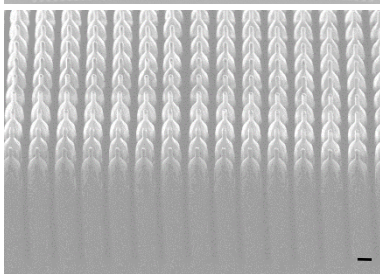
C



D



E



F

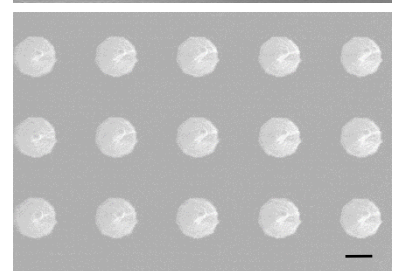
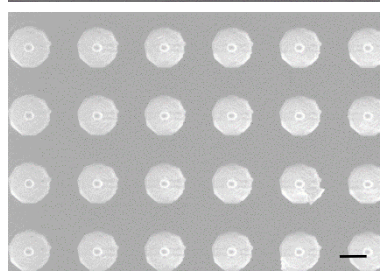
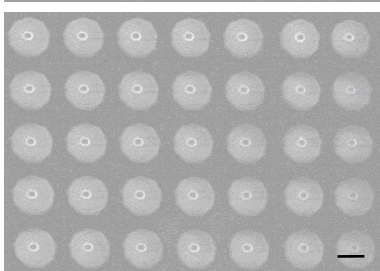


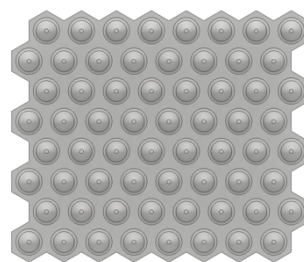
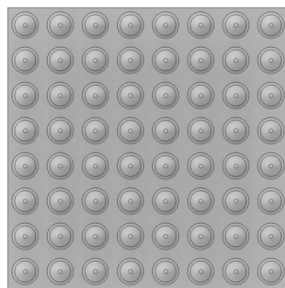
Table 2.1: Calculations of the different areas as well as the total number of features for each of the three different spacings.

Feature spacing	20 μm	25 μm	30 μm
Feature area	$20\ \mu\text{m} \times 20\ \mu\text{m} = 400\ \mu\text{m}^2$	$25\ \mu\text{m} \times 25\ \mu\text{m} = 625\ \mu\text{m}^2$	$30\ \mu\text{m} \times 30\ \mu\text{m} = 900\ \mu\text{m}^2$
Number of features in model	$10 \times 10 = 100$	$8 \times 8 = 64$	$6 \times 6 = 36$
Area of model	$400\ \mu\text{m}^2 \times 100 = 0.04\ \text{mm}^2$	$625\ \mu\text{m}^2 \times 64 = 0.04\ \text{mm}^2$	$900\ \mu\text{m}^2 \times 36 = 0.0324\ \text{mm}^2$
Array of model	$5 \times 5 = 25$	$5 \times 5 = 25$	$5 \times 5 = 25$
Area of array	$0.04\ \text{mm}^2 \times 25 = 1\ \text{mm}^2$	$0.04\ \text{mm}^2 \times 25 = 1\ \text{mm}^2$	$0.0324\ \text{mm}^2 \times 25 = 0.81\ \text{mm}^2$
Number of features in array	2500	1600	900

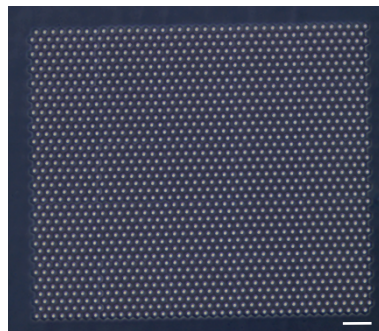
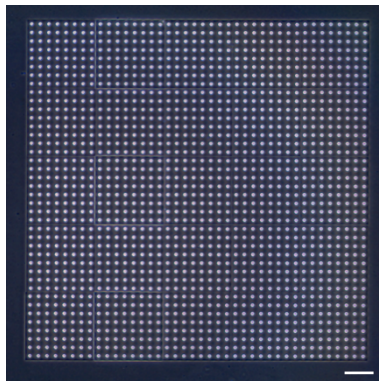
To increase the number of features within the same surface area, the array was further modified from a rectangular pattern to a hexagonal pattern. The 25 mm spacing design was drawn up with a hexagonal array and compared to the rectangular one. For a 1 mm² rectangular array, it is possible to print 1600 features, while for a hexagonal array, it is possible to fit 1840 features. This is due to the more compact packing of the same number of features within a smaller surface area when done in a hexagonal manner. This results in a 15% increase in terms of number of features, and ultimately the cell number within the same area. This also means that a 20 µm rectangular spacing is equivalent to a 23 µm hexagonal spacing in terms of feature numbers. These two different array variations were printed, imaged pre- and post- cleaning, and with the SEM (Figure 2.31). SEM images were taken by a PhD student in the group, Kun Zhou.

Figure 2.31: Images of the negative prints with two different array geometries. (A): 3D array of columns. (B) and (C): Light microscope images taken before (B) and after (C) the cleaning step, using an EVOS XL core microscope (Thermofisher). (D), (E) and (F): SEM image at 200X (D), 1000X at 30° tilt (E), and 2000X (F) magnification, taken by Kun Zhou using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bars: (B), (C) and (D): 100 µm, (E) and (F): 10 µm. The figure can be seen on the next page.

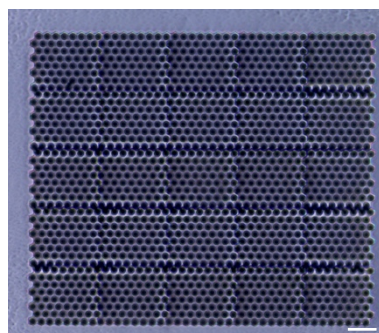
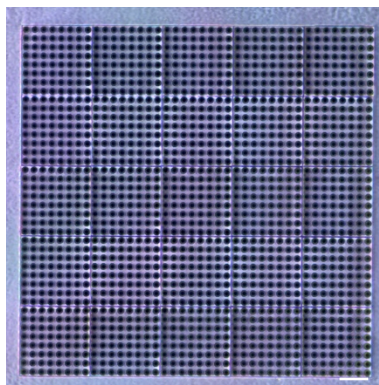
A



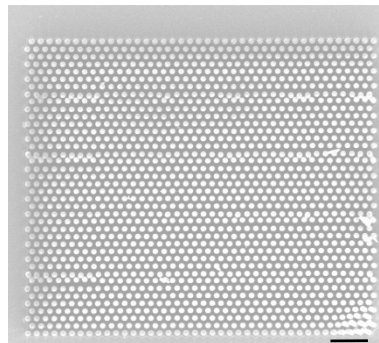
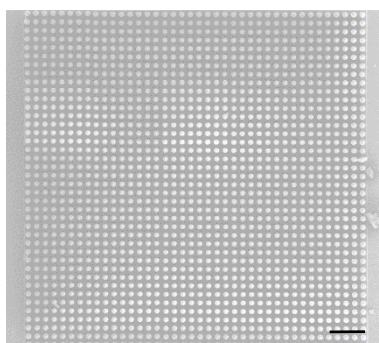
B



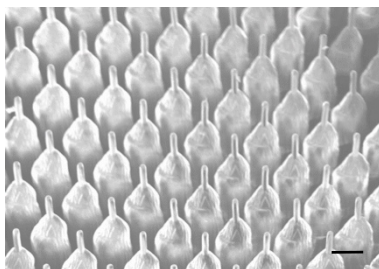
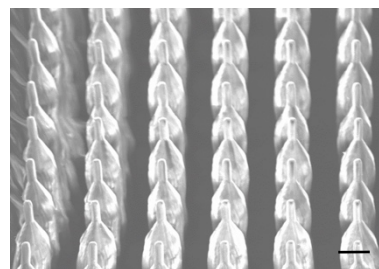
C



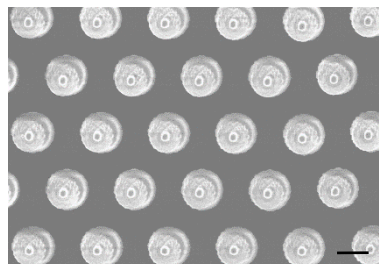
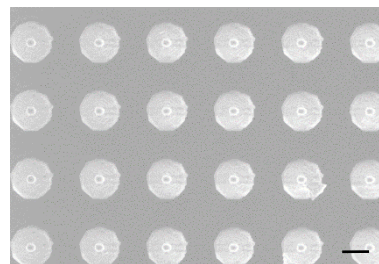
D



E



F



2.4.2 Modifying the print to a circle

Looking back at section 2.3.4, the second scaffold demoulding attempted tore as soon as it reached the edges of the printed negative mould. Therefore, to avoid stress concentrators and decrease the chances of the demoulded material tearing, sharp edges and corners in the 3D mould model were removed. This was achieved by changing the total print area from a square into a circle with a diameter of approximately 1 mm.

For these 3D models, the entire prints were drawn on the 3D software and then loaded into Describe to slice, hatch, and stitch the blocks together. Both the rectangular and the hexagonal arrays were drawn up, printed, and imaged as seen in Figures 2.32 and 2.33 below. In terms of the rectangular array, the blocks were easy to define, and the intersections were placed between the columns to make sure that each columns prints as one structure. However, this was not as intuitive with the hexagonal array, and a few of the columns ended up being printed along the intersection of two blocks. This can be seen in Figure 2.33 (D), and it is clear that the two blocks are not completely connected, and the structure is not a singular solid print.

The prints were successful at replicating the 3D model, however there are large deformities present in the print, in both array variations (Figures 2.32 and 2.33). The deformations are not located in any repeating location or pattern, therefore it was unclear the reason causing it. Knowing that previous issues with the print being damaged were caused by the cleaning step, adjusting the mould to fix this issue was the first thought.

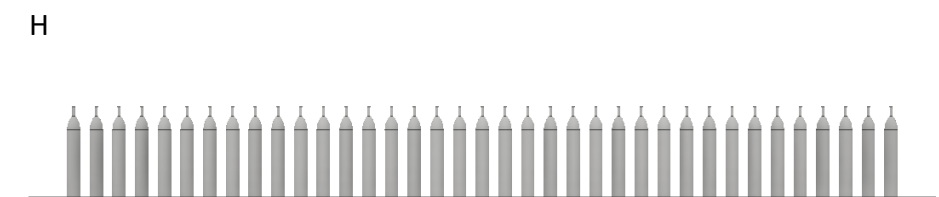
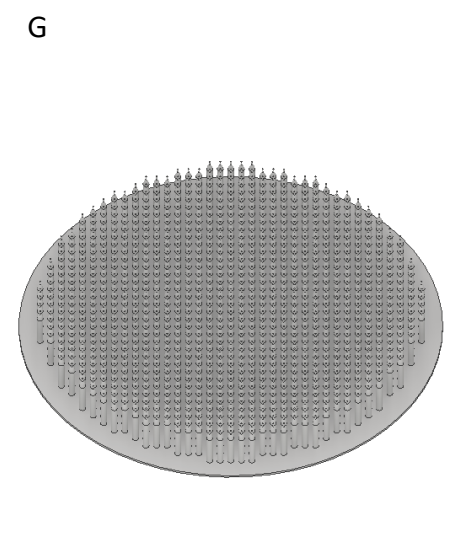
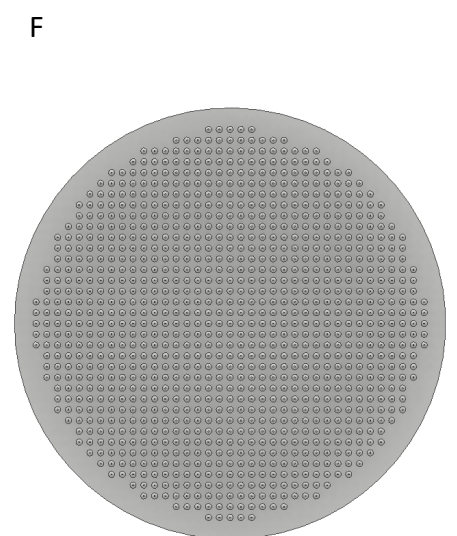
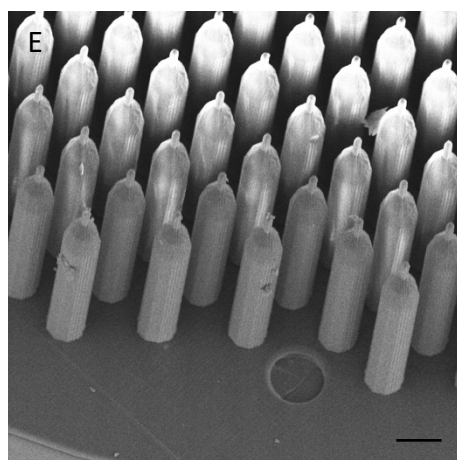
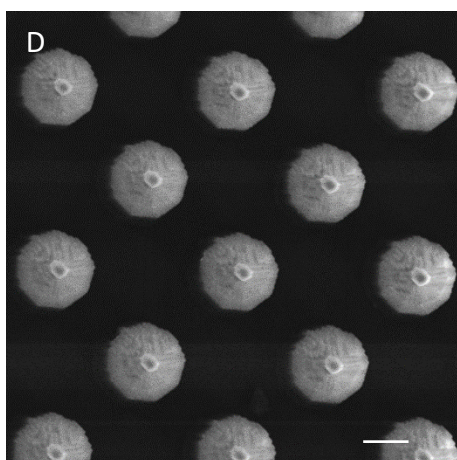
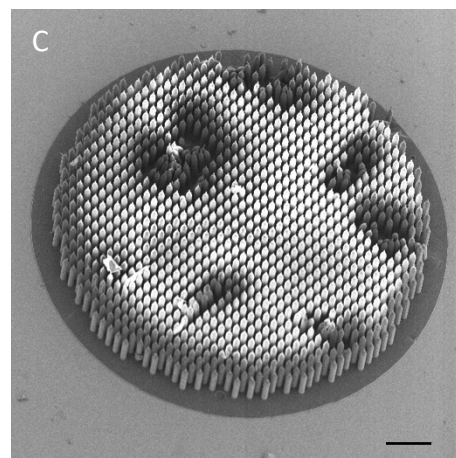
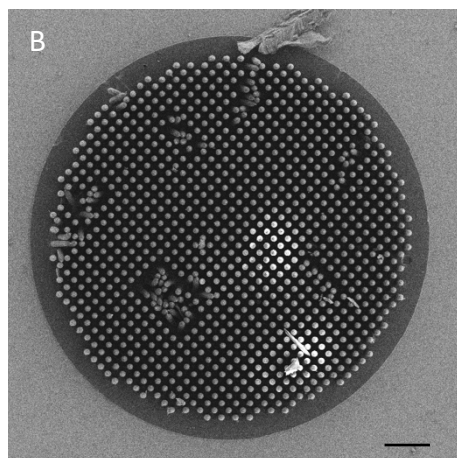
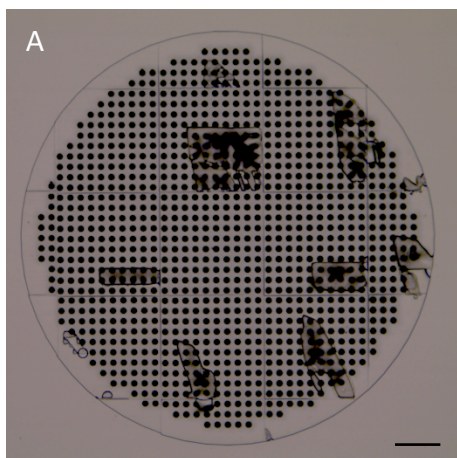


Figure 2.32: Circle area print with rectangular array.

(A): Light microscope image taken after the cleaning step, using an EVOS XL core microscope (Thermofisher).

(B): SEM image taken at 150X magnification.

(C): SEM image taken at 150X magnification and at 30° tilt.

(D): SEM image taken at 2000X magnification.

(E): SEM image taken at 1000X magnification and at 30° tilt.

(F): Top view of 3D model.

(G): Tilt view of 3D model.

(H): Side view of 3D model.

All SEM images were taken using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV.

Scale bars:

(A), (B) and (C): 150 μm

(D): 10 μm

(E): 20 μm

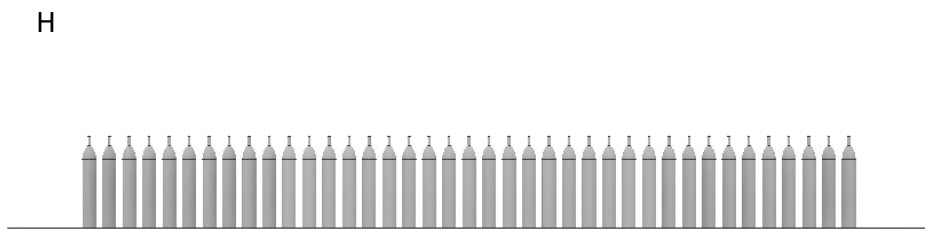
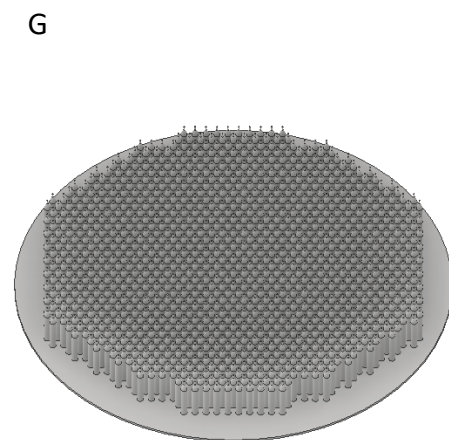
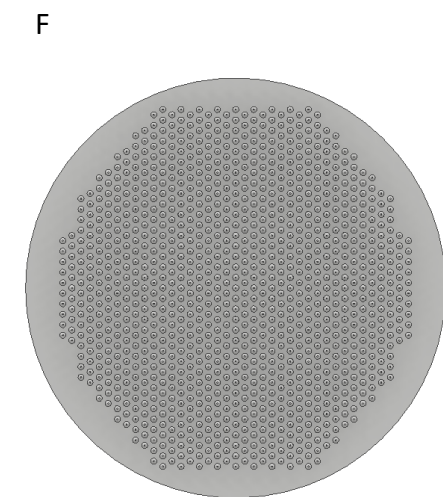
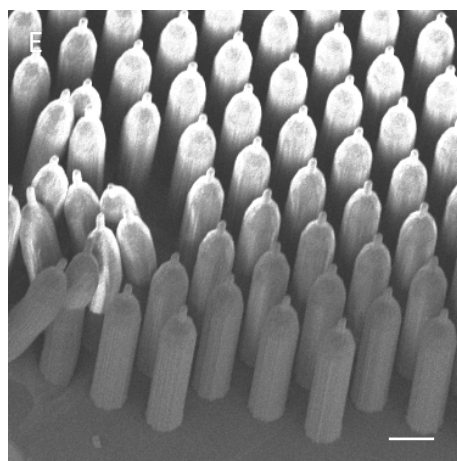
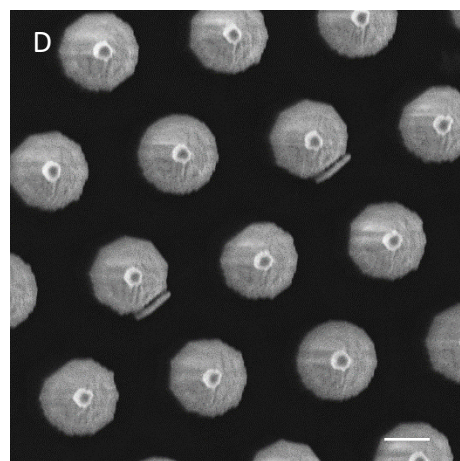
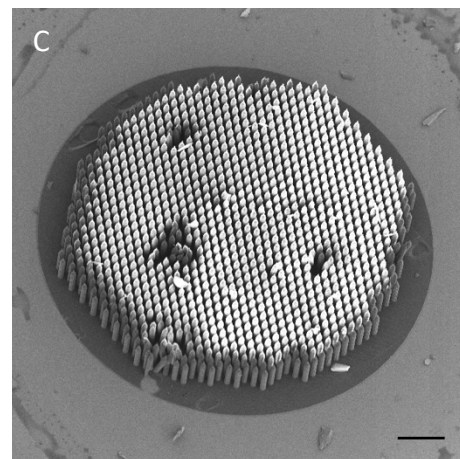
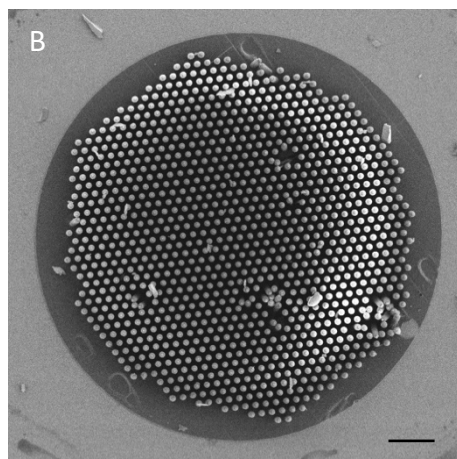
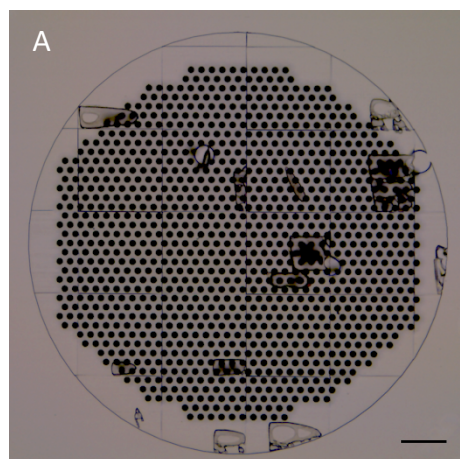


Figure 2.33: Circle area print with hexagonal array.

(A): Light microscope image taken after the cleaning step, using an EVOS XL core microscope (Thermofisher).

(B): SEM image taken at 150X magnification.

(C): SEM image taken at 150X magnification and at 30° tilt.

(D): SEM image taken at 2000X magnification.

(E): SEM image taken at 1000X magnification and at 30° tilt.

(F): Top view of 3D model.

(G): Tilted view of 3D model.

(H): Side view of 3D model.

All SEM images were taken using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV.

Scale bars:

(A), (B) and (C): 150 μm

(D): 10 μm

(E): 20 μm

2.4.3 Adjusting the 3D model to fix the deformities

In their 2020 paper, Wang *et al.* developed a superhydrophobic surface using nanostructures capable of repelling water [188]. However, these structures were fragile on their own, therefore they used a protective ‘armour’ to shield them. This armour consisted of a microstructure frame, surrounding the nanostructures, that provided robustness and durability. Even though their purpose was different and focused on enhancing the mechanical stability of their structures to make the surface superhydrophobic, their design inspired a modification for this print.

In order to protect the printed columns during the cleaning process, a wall surrounding the columns was added to the 3D model. The wall was designed to reach half the height of the features, making it 50 μm tall, as seen in Figure 2.34.

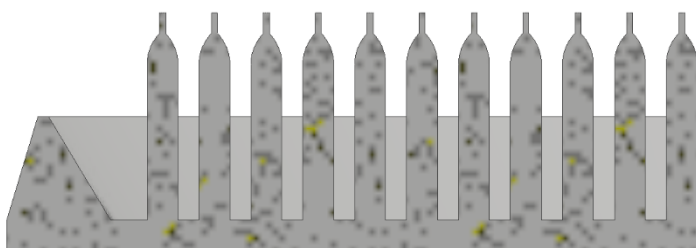


Figure 2.34: Modified 3D model. Cross section of the modified 3D model with added walls surrounding the columnar features.

Both the rectangular and the hexagonal arrays were drawn up, printed, and imaged as seen in Figure 2.34 and 2.36 below. Looking at the images, the deformities were still present in both array types, which suggests that the issue was not due to the cleaning step.

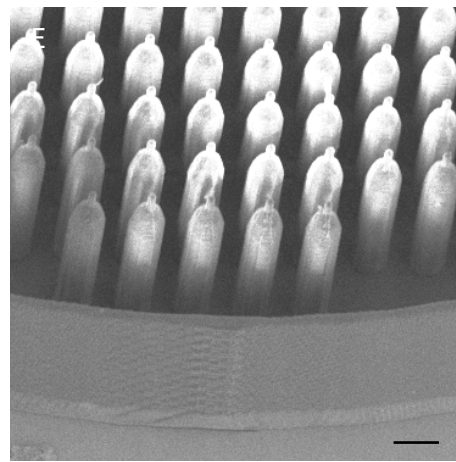
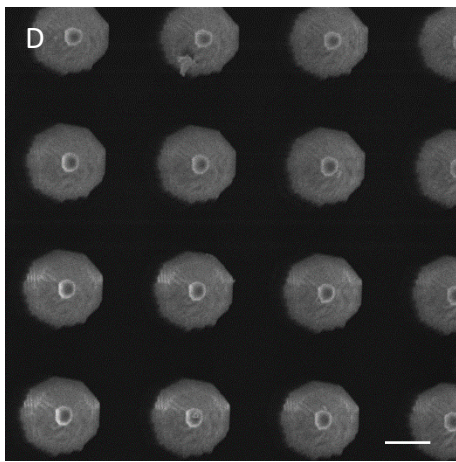
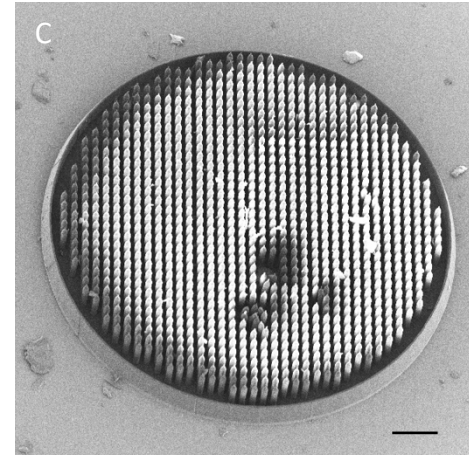
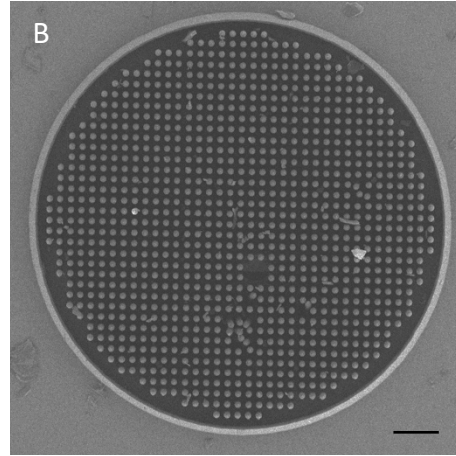
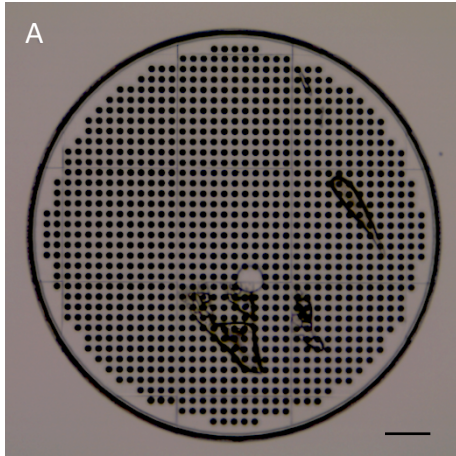


Figure 2.35: Circle area print with rectangular array with walls.

(A): Light microscope image taken after the cleaning step, using an EVOS XL core microscope (Thermofisher).

(B): SEM image taken at 150X magnification.

(C): SEM image taken at 150X magnification and at 30° tilt.

(D): SEM image taken at 2000X magnification.

(E): SEM image taken at 1000X magnification and at 30° tilt.

(F): Top view of 3D model.

(G): Tilted view of 3D model.

(H): Side view of 3D model.

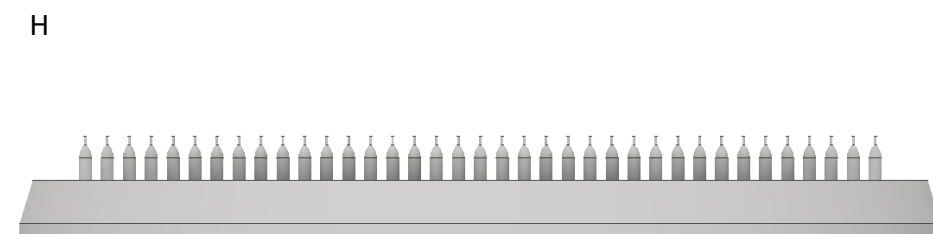
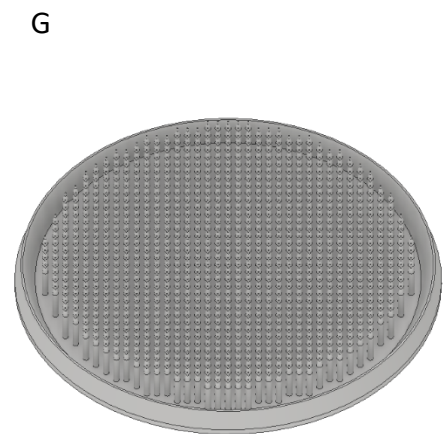
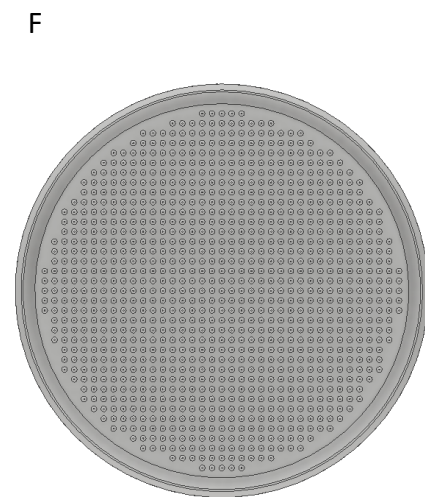
All SEM images were taken using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV.

Scale bars:

(A), (B) and (C): 150 μm

(D): 10 μm

(E): 20 μm



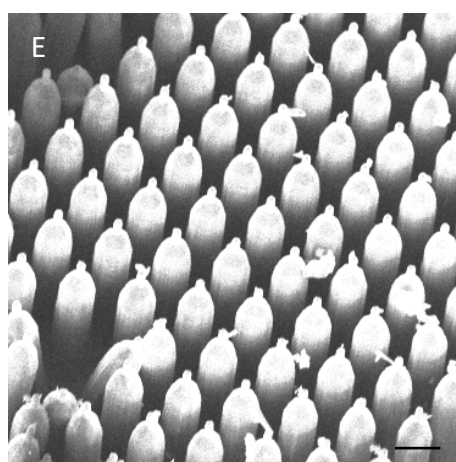
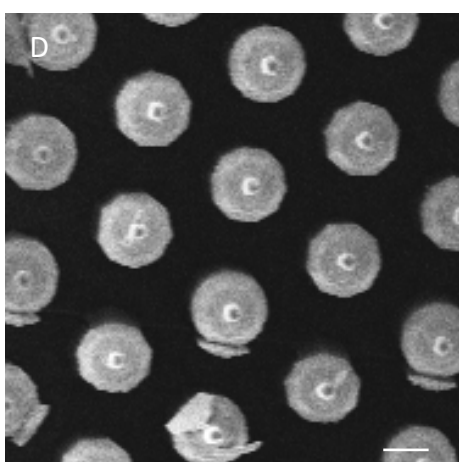
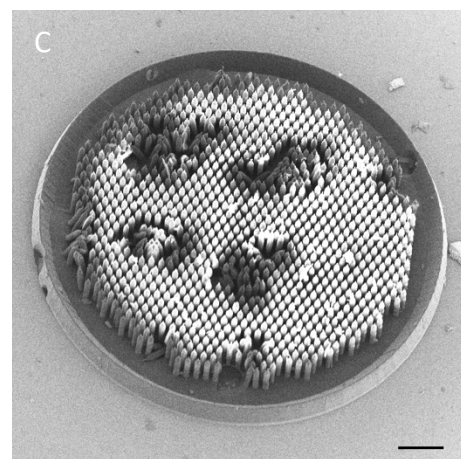
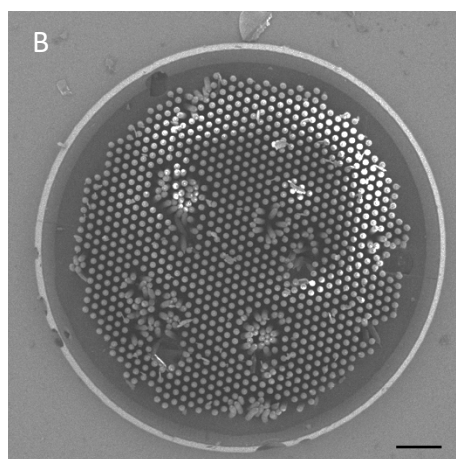
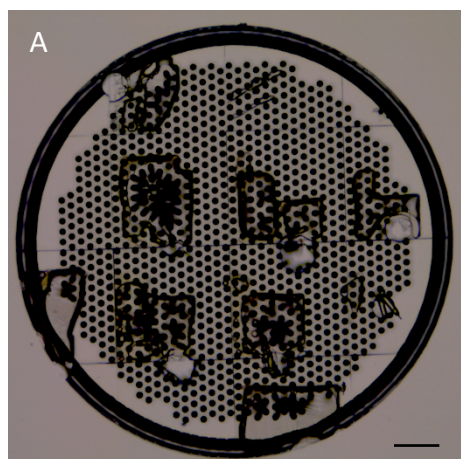


Figure 2.36: Circle area print with hexagonal array with walls.

(A): Light microscope image taken after the cleaning step, using an EVOS XL core microscope (Thermofisher).

(B): SEM image taken at 150X magnification.

(C): SEM image taken at 150X magnification and at 30° tilt.

(D): SEM image taken at 2000X magnification.

(E): SEM image taken at 1000X magnification and at 30° tilt.

(F): Top view of 3D model.

(G): Tilted view of 3D model.

(H): Side view of 3D model.

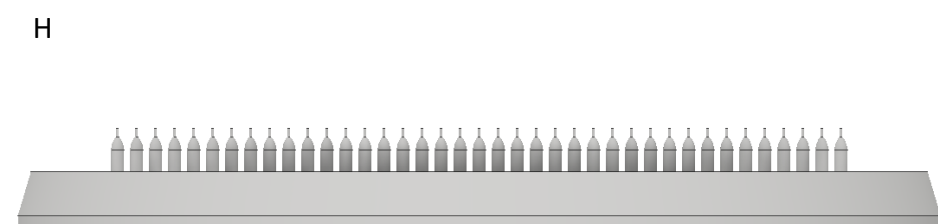
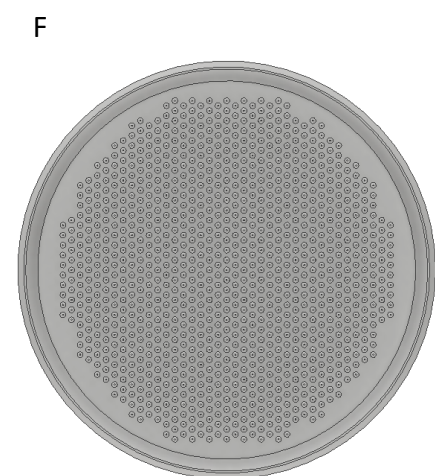
All SEM images were taken using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV.

Scale bars:

(A), (B) and (C): 150 μm

(D): 10 μm

(E): 20 μm



After consulting the Nanoscribe team, it was recommended to lower the power intensity setup on Describe to 70% (it was previously set at the default value of 100%), as the high power can cause an increase in the laser temperature, which in turn can trap air bubbles within the resin. Both arrays were printed, but only the hexagonal one is imaged below (Figure 2.37). This power adjustment solved the issue and there were no more deformities present in the print. Furthermore, to solve the issue of the columns being printed in two intersecting blocks in the hexagonal array, the spacing was increased to 35 μm , which resolved the issue.

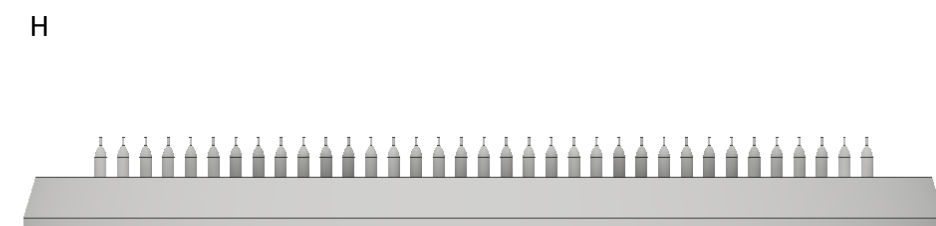
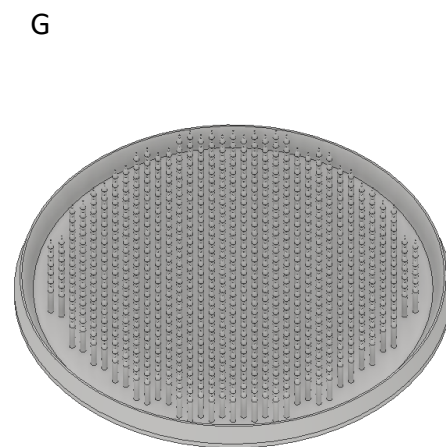
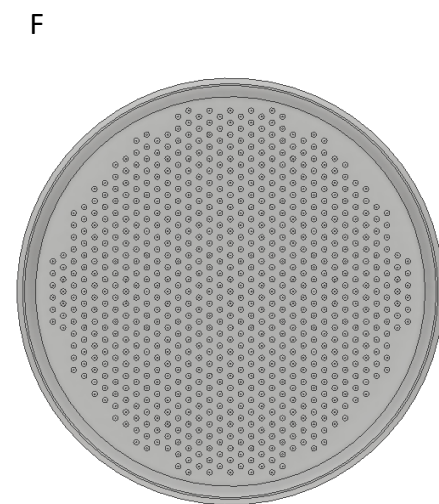
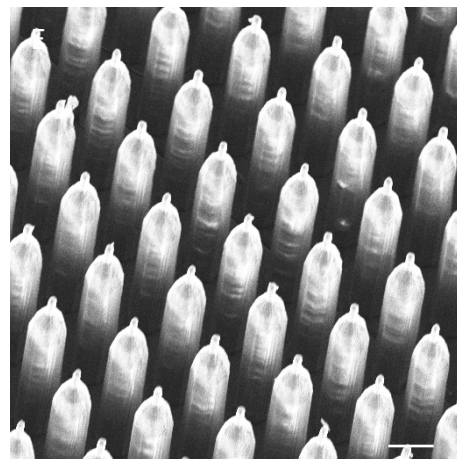
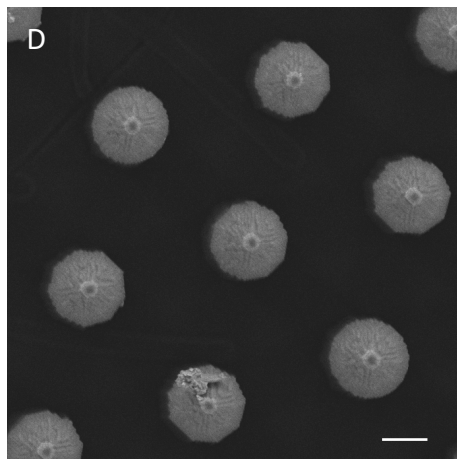
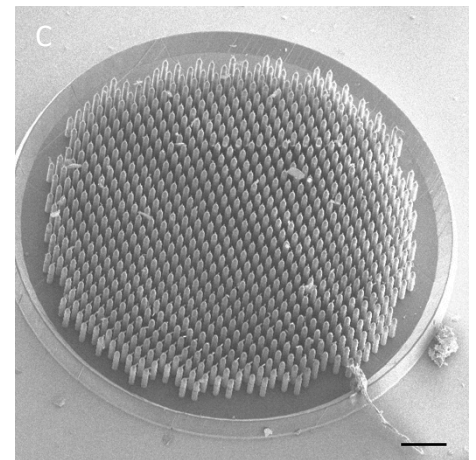
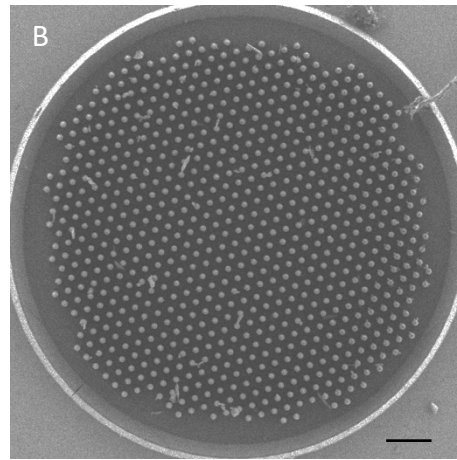
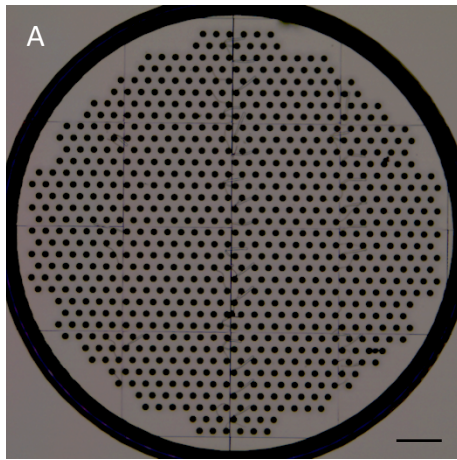


Figure 2.37: Circle area print with hexagonal array, walls and 70% power.

(A): Light microscope image taken after the cleaning step, using an EVOS XL core microscope (Thermofisher).

(B): SEM image taken at 150X magnification.

(C): SEM image taken at 150X magnification and at 30° tilt.

(D): SEM image taken at 2000X magnification.

(E): SEM image taken at 1000X magnification and at 30° tilt.

(F): Top view of 3D model.

(G): Tilted view of 3D model.

(H): Side view of 3D model.

All SEM images were taken using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV.

Scale bars:

(A), (B) and (C): 150 μm

(D): 10 μm

(E): 20 μm

2.4.4 Initial demoulding attempts with new circular 3D print

As previously mentioned, HAMA was chosen as an alternative biomaterial to the initial materials choice; GelMA. Hyaluronic acid is a biocompatible and biodegradable material that has been widely used in research pertaining to diseases of the eye [89], [90], [189]. Additionally, PEGDMA was added to the HAMA in order to produce a hydrogel with tuneable mechanical properties, decrease the swelling percentage [141] and tailor the degradation rate when exposed to hyaluronidase [161].

Following discussions with collaborators, it was determined that there was no clear value regarding how long the patterned hydrogel should remain in the retina post-transplantation *in vivo* to ensure the viability, integration, and interaction of the transplanted photoreceptor cells with the host retina. Therefore, instead of choosing one combination of HAMA-PEGDMA concentrations, a range of concentrations had to be determined, which would result in a wide range of degradation rates. The criteria used to narrow down this range was the ability to demould the hydrogel from the negative print.

The first concentrations chosen were arbitrary, but still relevant to what has been previously reported in the literature. The PEGDMA concentration was chosen to be 4% (w/v) and was kept constant for the experiments described in the rest of the Chapter. The HAMA concentration was initially chosen to be 6% (w/v), but the full range needed to be determined. The initiator chosen was LAP, and it was added at 0.05% (w/v), matching the earlier experiments conducted with GelMA.

The substrate and the print were prepared with the same process described above: it was plasma treated and silane treated, then a reservoir was created using a plastic sheet with a 6 mm punched hole. The mould used for the rest of the chapter was the 25 μm rectangular spacing mould with walls and printed with 70% laser power.

The gel precursor solution was prepared by weighing the lyophilised HAMA, PEGDMA and LAP into an Eppendorf tube and adding PBS to the polymer mixture. The tube was kept covered in aluminium foil to prevent any polymerisation from the surrounding light, and then vortexed to dissolve the LAP into the HAMA and PEGDMA.

In order to fabricate the scaffold, 20 μ L of the prepared gel precursor solution was added into the reservoir created around the negative print. The substrate was then placed on the spin coater for 10 minutes at 1000 RPM in order to spread the excess precursor solution away from the print, and only leave a layer with the thickness of the print. Once completed, a cover slip was added on top of the gel and the setup was placed under the UV lamp for 10 minutes to cure the gel. Once fully polymerised, the hydrogel, still sandwiched between the mould and the cover slip, was left to air dry for 30 minutes. Once the drying step was completed, the cover slip was removed, and with it, the hydrogel peeled off the print, while remaining stuck to the cover slip. The patterned hydrogel was imaged on a bright field microscope in air (Figure 2.38 (A) and (B)), and in PBS (Figure 2.38 (C)). The mould patterns were transferred successfully to the hydrogel, and there were almost no interconnected channels, meaning that increasing the spacing of the mould features was a successful modification. The scaffold was left in PBS for 24 hours, and was further imaged, as seen in Figure 2.39. The scaffold fabrication and the imaging were conducted by Dr. Eoghan Cunnane.

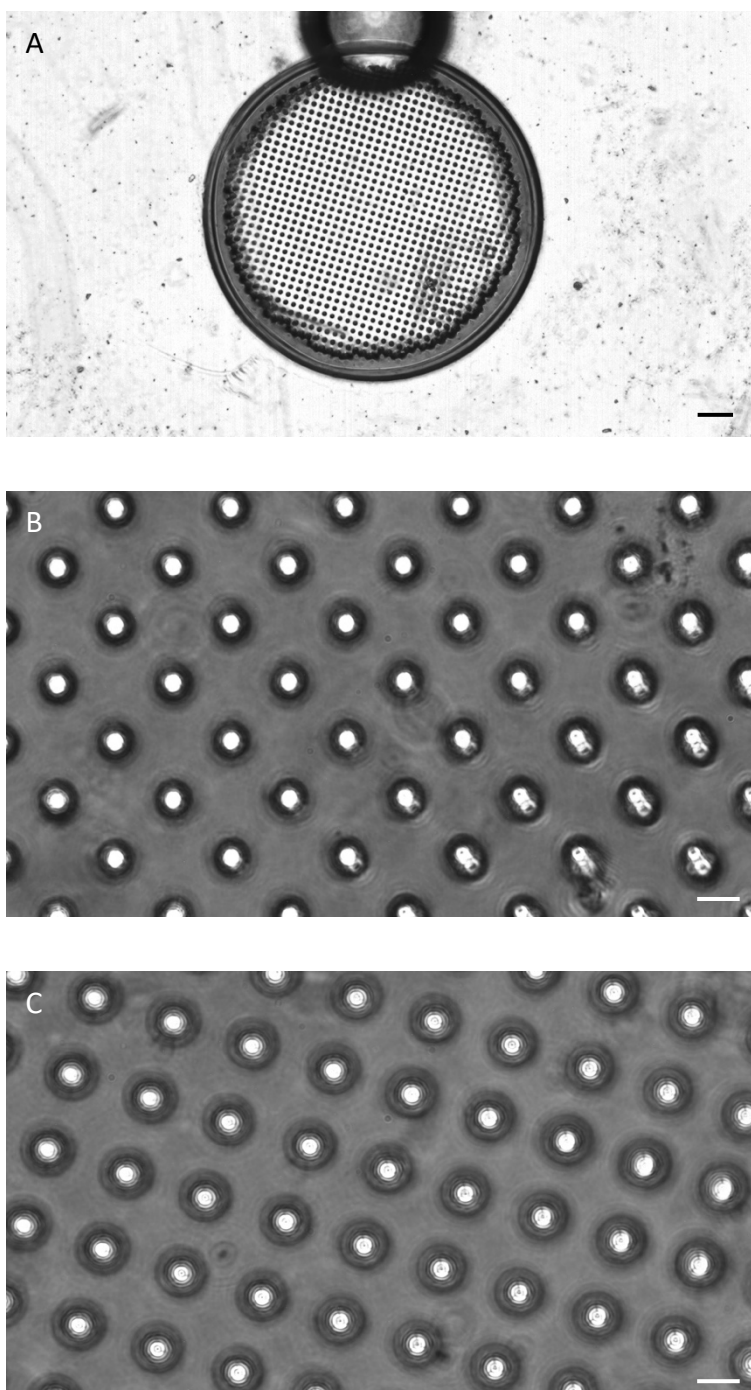


Figure 2.38: Patterned scaffold fabricated with 6% HAMA and 4% PEGDMA. (A) and (B) are images of the dry patterned scaffold at low (A) and high (B) magnifications, (C) is an image of the wet patterned scaffold. The scaffold was fabricated and imaged by Dr. Eoghan Cunnane. Scale bars: (A): 100 μm , (B) and (C): 10 μm .

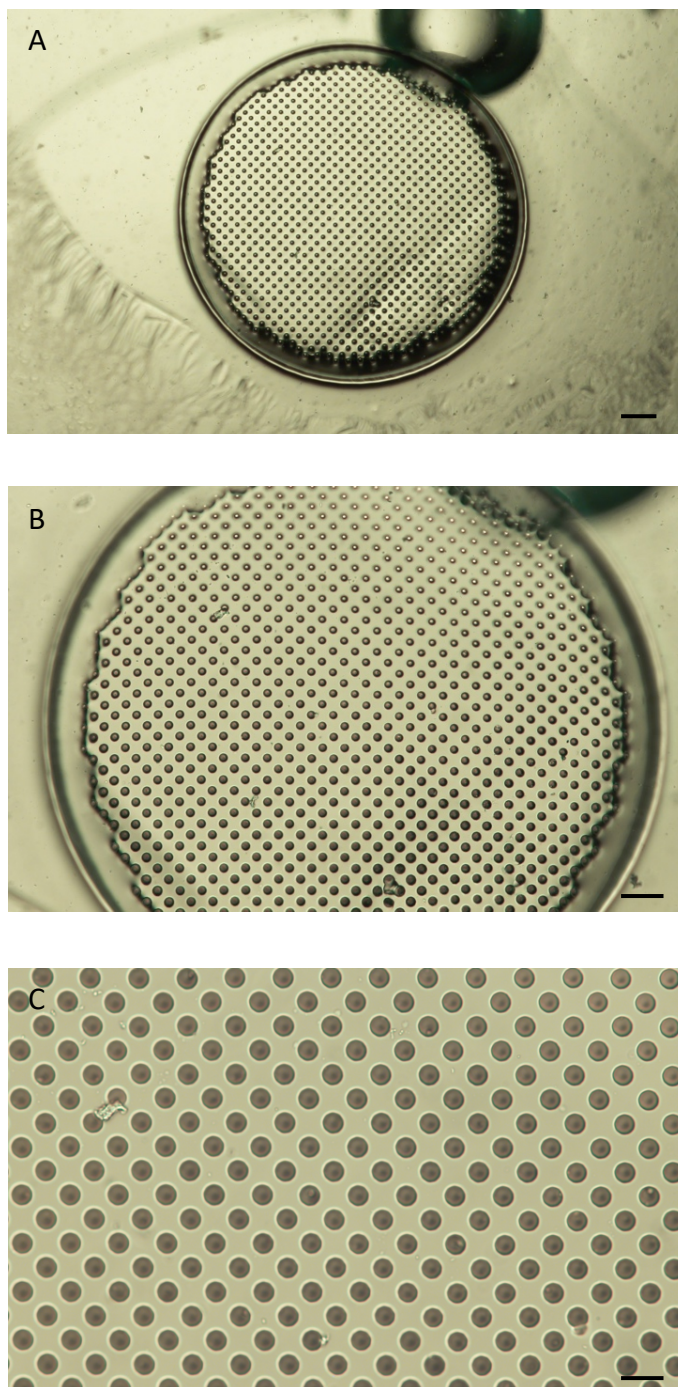


Figure 2.39: Patterned scaffold fabricated with 6% HAMA and 4% PEGDMA after 24 hours. Scaffold was imaged at different magnifications. The scaffold was fabricated and imaged by Dr. Eoghan Cunnane. Scale bars: (A): 100 μm , (B): 50 μm , (C): 20 μm .

With the success of the 6% HAMA – 4% PEG demoulding, the experiment was repeated with varying concentrations of HAMA, both higher and lower than 6%. The concentration values of HAMA tested can be found in Table 2.2 below. These experiments were conducted in collaboration with Dr. Eoghan Cunnane. It was found that with concentrations below 3%, it was not possible to demould the gel from the negative print, and with concentrations above 15%, the scaffold would tear. Therefore, it was decided that the range of HAMA concentrations to test was between 3% and 15%. This range of hydrogel concentrations, as well as more PEG and LAP concentrations, will be characterised and discussed much further in Chapter 3.

Table 2.2: The range of HAMA concentration values tested and their demoulding result.

0%	1.5%	3%	6%	9%	12%	15%	17.5%	20%
x	x	✓	✓	✓	✓	✓	x	x
Poor demoulding		Successful demoulding					Scaffold tearing	

Finally, after successfully demoulding hydrogels from the negative pattern, one of the gels was imaged using an upright confocal microscope. Z-stack images were taken by Dr. Eoghan Cunnane.

Unfortunately, the confocal images (Figure 2.40) revealed that even though the gel is demoulding and some of the features are transferred into the hydrogel, a large portion of the channel is not present, and the gels are much thinner than they should be. This meant that the gel precursor solution was not infiltrating the full depth of the negative mould. This demoulding process was optimised further, and all the modifications made will be discussed in Chapter 4.

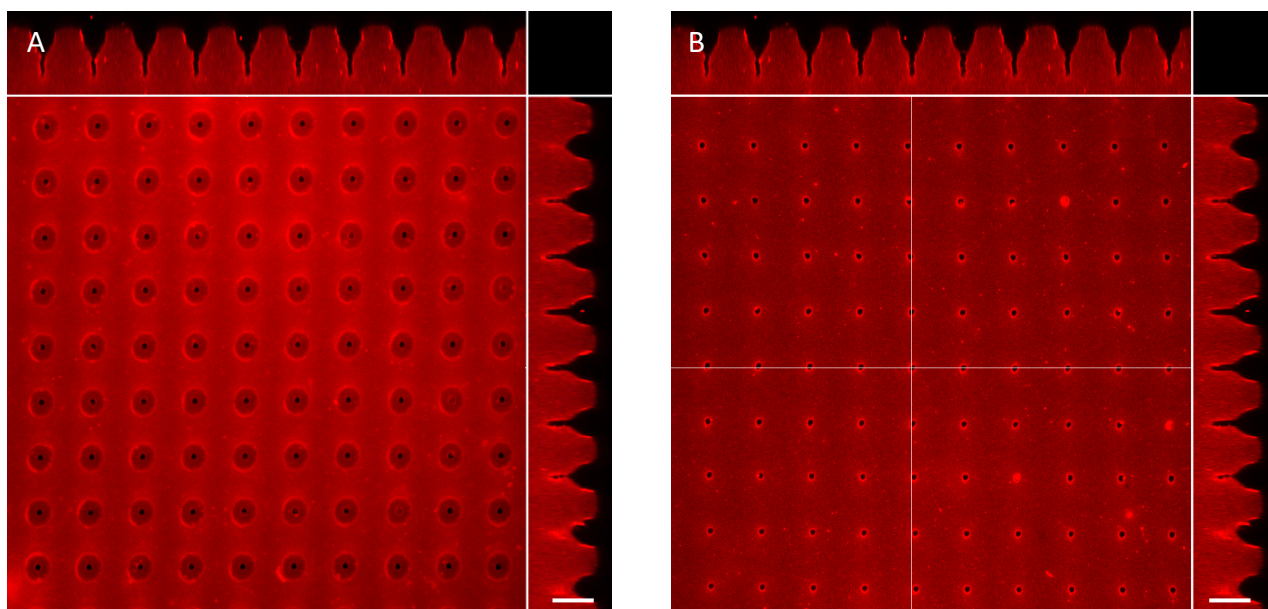


Figure 2.40: Confocal images of the demoulded patterned HAMA-PEGDMA scaffold. The images were taken from the top (A) and bottom (B) of the scaffold. The scaffold was fabricated and imaged by Dr. Eoghan Cunnane. Scale bar 20 μm .

2.5 Conclusion

The geometry of the scaffold developed in this chapter was modified from the ‘wine glass’ channel design in the Jung (2018) paper to a ‘champagne flute’ channel design, allowing the scaffold to hold a higher density of photoreceptor cells within each channel. As these larger channels could not be fabricated using the same photolithography methods, 3D printing technology was used. Using a high resolution two photon polymerisation printer, a highly defined negative print with columnar structures was fabricated. The printing process required some optimisation, including tuning the printing parameters and the cleaning process of the mould, as well as determining the best 3D design features of the mould such as spacing, total print height and array geometry. Although GelMA did show some promising results, HAMA was chosen instead for the initial demoulding attempts as well as the rest of the experiments in this thesis, due to several publications highlighting its positive role in increasing photoreceptor viability [42], [90], [92].

Chapter 3: Hydrogel characterisation: swelling, rheology, diffusion, and degradation studies

This micro-patterned hydrogel scaffold was designed for the purpose of polarizing photoreceptors in culture and to be transplanted into the subretinal space of diseased retinas. Therefore, it is crucial that the hydrogel is biodegradable and allows for particles to diffuse through it. Additionally, this hydrogel needs to be minimally swelling, as to preserve the micro-patterned channels. Further rheological testing can provide clearer understanding of how the hydrogel formulation affects the mechanical properties of the scaffold.

This chapter describes the hydrogel characterisation methods used to evaluate the swelling, rheological, diffusion, and degradation behaviours of the different formulations of HAMA – PEGDMA – LAP hydrogels and to determine the optimal polymer concentrations for the desired physical properties of the scaffold.

3.1 Swelling

Swelling ratio is defined by the percentage increase in the weight of the hydrogel submerged in a fluid due to water absorption. The capacity of a hydrogel to swell depends on several parameters, such as pore size, cross-linking density, and interactions between the polymers and the solvent. It is important to study the swelling ratio of a hydrogel in tissue engineering applications as it can define the gel's ability to both absorb body fluid after transplantation, in addition to allowing nutrient and waste transfer between the cells and the surrounding blood vessels [190].

In this project, the patterned hydrogel geometry and design are very specific. It is therefore vital that the swelling of the patterned gel is minimised to preserve the narrow channels and prevent blockage due to excess swelling. Thus, swelling tests were carried

out to understand how the varying concentrations of LAP, HAMA and PEGDMA in the hydrogel formulation influences the swelling properties of the hydrogel, and to identify the formulation that minimises swelling.

3.1.1 Background

Swelling tests are conducted extensively in the published literature because of their importance in characterising hydrogels for tissue engineering. Some papers are discussed below to understand the different approaches that are used to measure swelling.

Oudshoorn *et al.* 2007 synthesised methacrylated hyaluronic acid with various degrees of functionalisation that could be precisely controlled, ranging from 5 to 30% [146]. The swelling ratio of the HAMA hydrogel was determined for 5 different degrees of functionalisation by weighing them directly after their polymerisation at time 0 (W_0), and then weighing them again at regular time intervals (W_t). The swelling ratio was calculated by dividing the wet weight at each time point by the dry weight at time point 0:

$$Swelling\ ratio = \frac{W_t}{W_0}$$

It was found that the swelling ratio of the hydrogels decreased with increasing DoF, and that for values of DoF above 15%, there was almost no swelling.

Noh *et al.* 2019 studied the swelling of printable bioink hydrogels, consisting of hyaluronic acid, hydroxyethyl acrylate and gelatin methacryloyl [191]. In their paper, the authors evaluated the swelling percentage of their gels by first drying the gels and measuring their dry weight (W_{dry}), and then rehydrating them and measuring their wet weight (W_{wet}) at regular time intervals (1 hour). To obtain the most accurate wet weight measurements, the excess surface water on the hydrogels was blotted off using a tissue paper. The swelling ratio percentage was calculated using the following formula:

$$Swelling (\%) = \frac{W_{wet} - W_{dry}}{W_{dry}} \times 100\%$$

They reported swelling over a total of 11 hours, with a final swelling percentage of around 8000%, and they attributed that large swelling value to the various hydrophilic functional groups present in their hydrogels.

Masters *et al.* (2005) studied the swelling behaviour of HAMA as a biomaterial for valvular interstitial cell culture [141], [190]. Their experiments were conducted similarly to those described in Noh *et al.* (2019), and the swelling percentage was determined using the same formula, however they studied swelling not only for HAMA gel, but also for HAMA co-polymerised with 5% and 10% PEG diacrylate (PEGDA), and over a range of HAMA degrees of methacrylation and PEGDA concentrations. It was determined that the swelling percentage decreases with increasing HAMA methacrylation. Furthermore, the swelling percentage of HAMA decreased four-fold with 5% PEGDA and 7 folds with 10% PEGDA when compared to HAMA on its own.

Velasco-Rodriguez *et al.* (2021) also studied the effect of combining HAMA with another polymer [190]. They characterised the swelling behaviour of pure or hybrid GelMA-HAMA gels, at several different concentrations. The swelling degree was calculated using the same methods described in Noh *et al.* (2019). Pure and hybrid hydrogels displayed decreasing swelling with increasing polymer concentrations, with a high swelling rate during the first 90 minutes followed by a more stable swelling rate after 10 h for gels containing pure HAMA.

Rizwan *et al.* (2017) used the same methods described in Noh *et al.* (2019) to determine the degree of swelling (%) of their GelMA hydrogels [112]. They did not study the evolution of swelling over time, instead they compared the effect of various concentrations on swelling behaviour. The gels were lyophilised after they were cured to

measure their dry weight and then were rehydrated overnight before their wet weight was measured the following day. It was reported that the degree of swelling decreased with increasing concentration.

Podervaat *et al.* (2017) used HAMA as a biomaterial for bone constructs in bone regenerative medicine [124]. The hydrogels were polymerised, and their weight was directly measured at time 0 (W_0). Following that, they were submerged in PBS and weighed at multiple time points to get their wet weight (W_{wet}). The experiment was conducted for a total of three weeks. The swelling ratio was calculated by dividing the wet weight by the dry weight:

$$Swelling (\%) = \frac{W_{wet}}{W_0} \times 100\%$$

The hydrogel wet weight increased over 3 weeks, however the largest weight increase happened during the first 24 hours of incubation. Furthermore, it was found that the swelling was the highest for the lowest concentration hydrogels and decreased with increasing gel concentration. It can be concluded from the preceding literature that swelling decreases with increasing polymer concentrations, and also with addition of a co-polymer such as PEG.

3.1.2 Materials and methods

The hydrogels characterised in this section were prepared using synthesised HAMA and PEGDMA, as described in Chapter 2. Lyophilized HAMA and PEGDMA were dissolved in 1X PBS containing 1% LAP to prepare the precursor solution. The LAP solution was first added to the PEGDMA and vortexed until the PEGDMA was fully dissolved. Following that, the new solution mixture was pipetted on top of the HAMA and left overnight at 4°C to dissolve the lyophilized HAMA. The following day, 40 μ L of the HAMA-PEGDMA precursor solution was pipetted into 1 mL syringes, with the top already

cut off, and crosslinked for 15 minutes under a 365 nm UV lamp. This setup provided reproducible cylindrical hydrogels and was used for all the swelling experiments.

As there were three parameters to study for each formulation, two parameters were kept constant as the third was swept across five (or six in the case of LAP) different concentrations. The studied formulations are described in Table 3.1 below. All the experiments were performed in triplicate.

Table 3.1: Combinations of HAMA-PEGDMA-LAP concentrations chosen for the three swelling experiments. The first was to screen LAP concentrations, the second to screen PEGDMA concentrations and the last was to screen the HAMA concentrations.

	HAMA (%)					PEGDMA (%)					LAP (%)					
1	Constant					Constant					Variable					
	9					0					0.05	0.1	0.5	1	5	10
2	Constant					Variable					Constant					
	9					0	2	4	8	12	1					
3	Variable					Constant					Constant					
	3	6	9	12	15	4					1					

In this study, the goal was focused on understanding swelling due to water absorption from the gel directly following its curing. However, the swelling ratio of dry to swollen gel was also studied. The weight measurements were performed at the following time points: 0 minutes (this measurement was performed immediately after the gels were fully cured, and with minimal wait as to not allow for evaporation or drying of the gels), 1 hour, 24 hours and 10 days (240 hours). The hydrogels were transferred one at a time into pre-

weighed Eppendorf tubes and placed on a scale to record their weight. The tubes were then filled with 1 mL of milliQ water and left in the incubator for the relevant amount of time. For each subsequent time point, the gels were taken out of the tube with a spatula, and lightly dabbed with a tissue to absorb any excess water on the surface of the gels (Noh 2019). They were then weighed in a new pre-weighed Eppendorf tube and the process was repeated until the final time point. Finally, the gels were frozen and lyophilized and the final weight of the dry gel was measured and recorded to calculate the swelling ratio.

The swelling ratio represents the fractional increase of the weight of a hydrogel due to water uptake. However, it is a comparison of the total amount of water a gel can take up in contrast to the dry gel containing no water. The calculations for each combination of parameters were performed using the following equations:

$$\text{Water uptake (\%)} = \frac{M_t - M_{t=0}}{M_t} \times 100$$

$$\text{Swelling ratio} = \frac{M_S - M_D}{M_D}$$

The final values plotted for the water uptake curves are the mean of the measurements across the three gel replicates per condition as well as the standard deviation of the measurements. The values plotted for the swelling ratio graphs are the three replicate gel measurements per condition as well as the mean of the measurements.

3.1.3 Results and discussion

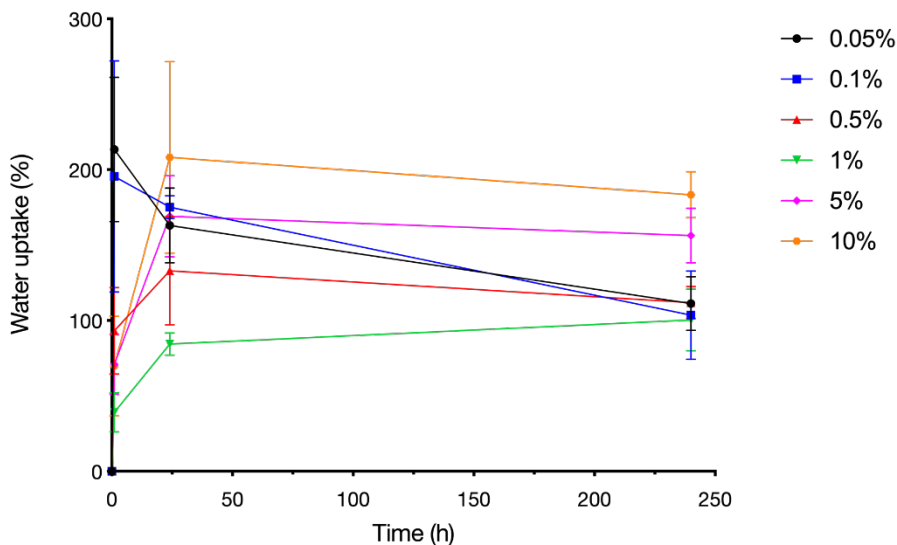
The figures below show the progression of the water uptake for the hydrogels over time for the different experiments and formulations. To understand the variations and significant differences in measurements for swelling, a two-way Anova test was carried out including Tukey's multiple comparisons test, to compare the combination of

concentrations two at a time. The results of the statistical analysis tests are also reported in tables for each parameter screening.

3.1.3.1 Screening the LAP concentration

The first experiment consisted of a LAP concentration screening, as seen in Figure 3.1 (A). This figure shows how the hydrogel swelling behaviour evolves over time for hydrogels with fixed HAMA and PEGDMA concentrations, but for varying LAP concentrations. The data was split into two graphs, the first one containing the two lowest concentrations, and the second four higher ones, as seen in Figure 3.1 (B).

A



B

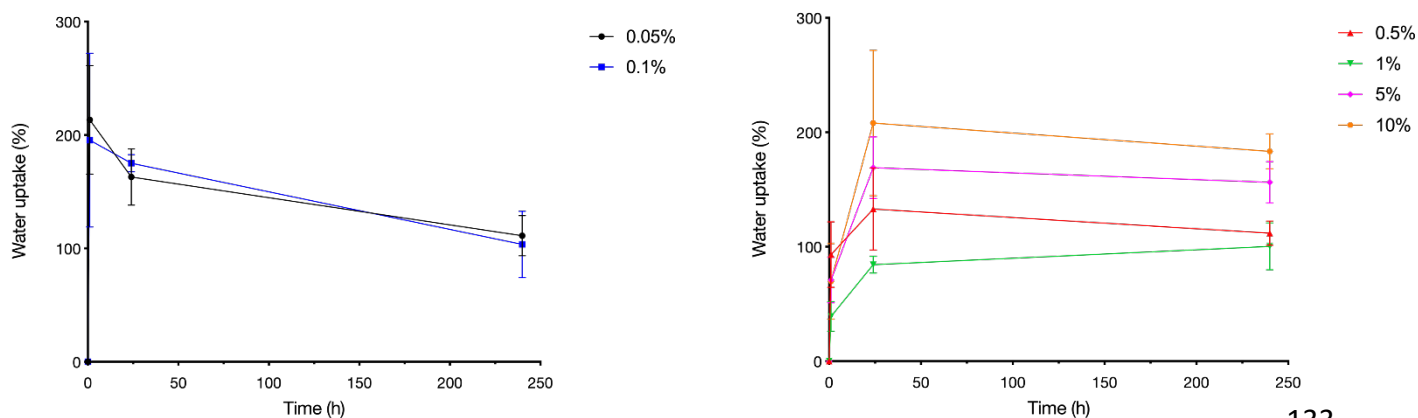


Figure 3.1: Percentage of hydrogel water uptake over time for different LAP concentrations. The data was plotted together (A) and divided into 0.05% and 0.1% on the left graph, and 0.5%, 1%, 5% and 10% on the right graph (B) (n=3). The figure can be seen on the previous page.

Table 3.2: Tukey's multiple comparisons test (Two-way Anova) comparing the combination of two concentrations at a time for values in Figure 3.1.

Tukey's multiple comparisons test	Significance summary	Adjusted P value
0.05% vs. 0.1%	ns	0.9997
0.05% vs. 0.5%	*	0.0275
0.05% vs. 1%	****	<0.0001
0.05% vs. 5%	ns	0.3826
0.05% vs. 10%	ns	0.9928
0.1% vs. 0.5%	ns	0.0568
0.1% vs. 1%	****	<0.0001
0.1% vs. 5%	ns	0.5588
0.1% vs. 10%	ns	0.9998
0.5% vs. 1%	ns	0.1622
0.5% vs. 5%	ns	0.8113
0.5% vs. 10%	ns	0.1078
1% vs. 5%	**	0.0071
1% vs. 10%	****	<0.0001
5% vs. 10%	ns	0.7323

The first graph contains the lower two concentrations of LAP: 0.05 and 0.1%. A 200% increase in water uptake percentage is observed during the first hour of incubation, followed by a continuous decrease in the percentage of water uptake of the gels over the remaining 10 days, caused by a decrease in the mass of the gels. The most likely interpretation is that the LAP concentrations were too low to fully crosslink the gels, therefore the initial network was not very densely crosslinked, allowing water to be

absorbed in a large amount fast. Following that, the un-crosslinked HAMA and PEGDMA materials were released from the gel into the PBS over time, which explains the decrease in mass. Due to this, water uptake percentage cannot be correctly interpreted from the data.

The second graph contains the remaining four concentrations of LAP: 0.5%, 1%, 5%, 10%. There was a very fast increase in water uptake over the first hour for all conditions. However, the water uptake percentage of the gels kept increasing over time. In all four conditions, the water uptake (and thus the mass) increased after the first 24 hours and then reached a more sustained phase until the end of the experiment at 10 days. From the graph, 1% LAP gels had taken up the least amount of water overall over time and swelled the least.

Taking into consideration Tukey's multiple comparisons test, as seen in Table 3.2, the swelling behaviours of all the concentrations are significantly different to that of 1% LAP, therefore it can be concluded that the 1% LAP gels swell significantly less than the other concentrations and 1% is the most appropriate concentration of LAP to proceed with in the following tests.

3.1.3.2 Screening the PEGDMA concentration

The second experiment consisted of a PEGDMA concentration screening, as seen in Figure 3.2. This figure shows how the hydrogel swelling behaviour evolves over time for hydrogels with fixed HAMA and LAP concentrations, but for varying PEGDMA concentrations. In all conditions, there was a very fast increase in water uptake over the first hour of incubation. The percentage of water uptake then reached a more sustained phase for all concentrations until the end of the experiment at 10 days. From the graph, the percentage of water uptake decreased as the PEGDMA concentration increased, with a total water uptake almost halved from 0% to 12% PEGDMA.

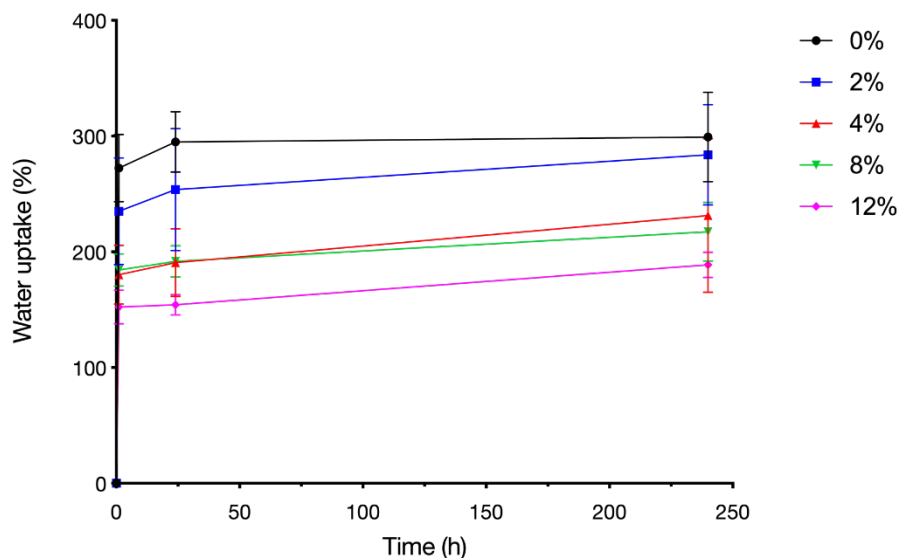


Figure 3.2: Percentage of hydrogel water uptake over time for different PEGDMA concentrations (n=3). For all conditions, the water uptake increases fast over the first hour, then reaches a sustained phase for the rest of the experiment.

Taking into consideration Tukey's multiple comparisons test, seen in Table 3.3, increasing the concentration from 4% to 8% and to 12% PEGDMA did not have a significant effect on swelling. However, increasing it from 0% to 4% and 2% to 4% did decrease the water uptake percentage significantly. Therefore, it can be concluded that the 4% PEGDMA gels display a lower amount of swelling when compared to 0% and 2% and is the most appropriate concentration of PEGDMA to proceed with for the final experiment.

The swelling ratio analysis in Figure 3.3 and Table 3.4, reveals the swelling ratio of the different gels did not vary significantly with increasing PEGDMA concentration, except in comparison to the 0% PEGDMA.

Therefore, it can be concluded that the addition of PEGDMA increases the crosslinking density within the hydrogel no matter what concentration of PEGDMA is added to the HAMA [141].

Table 3.3: Tukey's multiple comparisons test (Two-way Anova) comparing the combination of two concentrations at a time for values in Figure 3.2.

Tukey's multiple comparisons test	Significance summary	Adjusted P value
0% vs. 2%	ns	0.3046
0% vs. 4%	****	<0.0001
0% vs. 8%	****	<0.0001
0% vs. 12%	****	<0.0001
2% vs. 4%	**	0.0081
2% vs. 8%	**	0.0049
2% vs. 12%	****	<0.0001
4% vs. 8%	ns	0.9997
4% vs. 12%	ns	0.1882
8% vs. 12%	ns	0.2610

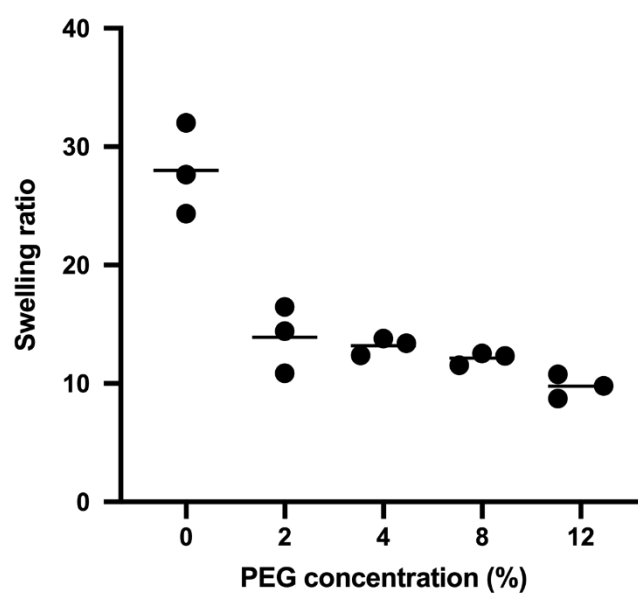


Figure 3.3: Swelling ratios of different PEGDMA concentrations (n=3). The swelling ratios of the different concentrations did not vary significantly with increasing PEGDMA concentration except in comparison to the 0% PEGDMA.

Table 3.4: Tukey's multiple comparisons test (Two-way Anova) comparing the combination of two concentrations at a time for values in Figure 3.3.

Tukey's multiple comparisons test	Significance summary	Adjusted P value
0% vs. 2%	***	0.0001
0% vs. 4%	****	<0.0001
0% vs. 8%	****	<0.0001
0% vs. 12%	****	<0.0001
2% vs. 4%	ns	0.9937
2% vs. 8%	ns	0.8610
2% vs. 12%	ns	0.2272
4% vs. 8%	ns	0.9762
4% vs. 12%	ns	0.3846
8% vs. 12%	ns	0.6925

3.1.3.3 Screening the HAMA concentration

The third experiment consisted of a HAMA concentration screening, as seen in Figure 3.4. This figure shows how the hydrogel swelling behaviour evolves over time for hydrogels with fixed PEGDMA and LAP concentrations, but for different PEGDMA concentrations. In all conditions, there was a very fast increase in water uptake over the first hour of incubation. The percentage of water uptake then reached a more sustained phase for all concentrations until the end of the experiment at 10 days. Apart from the 3% HAMA, the other concentrations did not display significant differences in terms of total water uptake over the course of the experiment.

Analysing the data using Tukey's multiple comparisons test, seen in Table 3.5, increasing the concentration from 6% to any of the other concentrations does yield a significant difference of water uptake percentage (apart from a slight significance between 6% and 15%).

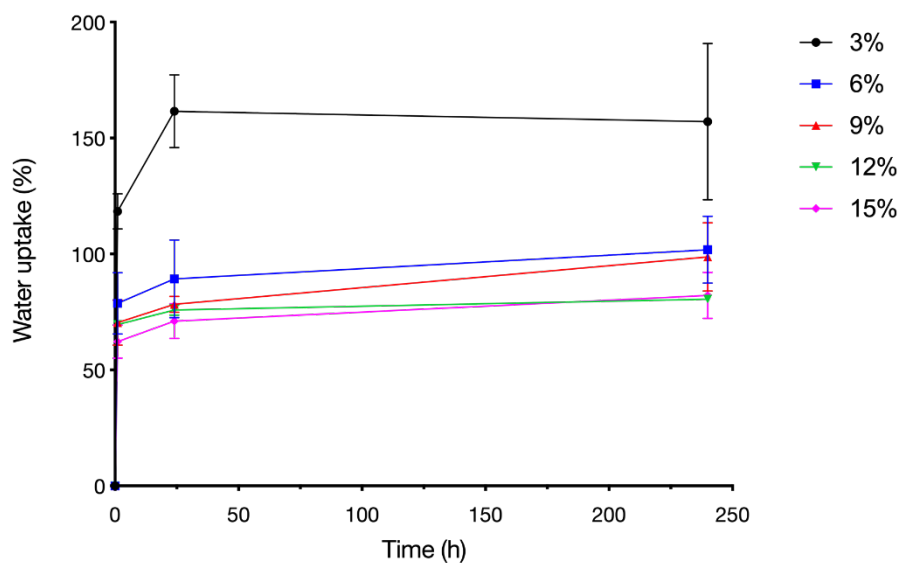


Figure 3.4: Percentage of hydrogel water uptake over time for different HAMA concentrations (n=3). For all conditions, the water uptake increases fast over the first hour, then reaches a sustained phase for the rest of the experiment. The percentage of water uptake does not vary significantly except in comparison to the 3% HAMA.

Table 3.5: Tukey's multiple comparisons test (Two-way Anova) comparing the combination of two concentrations at a time for values in Figure 3.4.

Tukey's multiple comparisons test	Significance summary	Adjusted P value
3% vs. 6%	****	<0.0001
3% vs. 9%	****	<0.0001
3% vs. 12%	****	<0.0001
3% vs. 15%	****	<0.0001
6% vs. 9%	ns	0.7610
6% vs. 12%	ns	0.1578
6% vs. 15%	*	0.0463
9% vs. 12%	ns	0.7826
9% vs. 15%	ns	0.4421
12% vs. 15%	ns	0.9797

The swelling ratio analysis in both Figure 3.5 and Table 3.6, reveals that the swelling ratio of the different gels did vary significantly with increasing HAMA concentration in comparison to the 3% and 6% HAMA (except in the case of 6% and 9%). Therefore, it can be concluded that increasing the concentration of HAMA does increase the crosslinking density within the hydrogel and decreases the swelling. Furthermore, as there is no significant difference in swelling when increasing the HAMA concentration from 9% to 12% and 15%, it appears that 9% might be a good choice of HAMA concentration to use for the optimisation steps in Chapter 4.

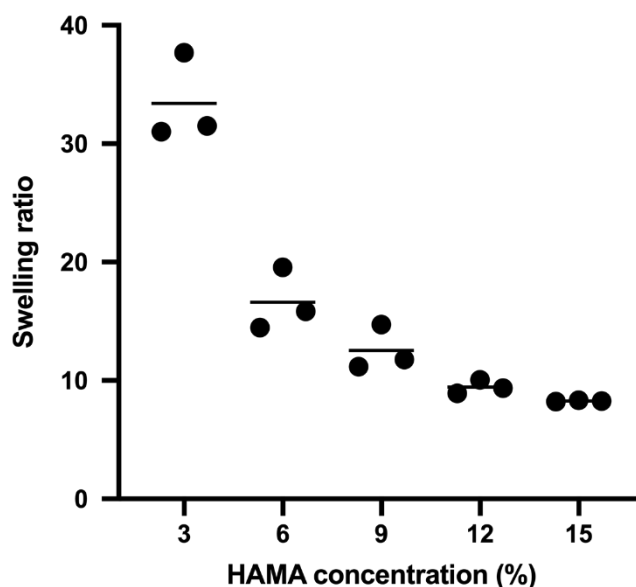


Figure 3.5: Swelling ratio of different HAMA concentrations (n=3). The swelling ratios of the different concentrations did not vary significantly with increasing HAMA concentration in comparison to the 3% and 6% HAMA (except in the case of 6% and 9%).

Table 3.6: Tukey's multiple comparisons test (Two-way Anova) comparing the combination of two concentrations at a time for values in Figure 3.5.

Tukey's multiple comparisons test	Significance summary	Adjusted P value
3% vs. 6%	****	<0.0001
3% vs. 9%	****	<0.0001
3% vs. 12%	****	<0.0001
3% vs. 15%	****	<0.0001
6% vs. 9%	ns	0.2413
6% vs. 12%	*	0.0180
6% vs. 15%	**	0.0068
9% vs. 12%	ns	0.4690
9% vs. 15%	ns	0.2046
12% vs. 15%	ns	0.9638

3.2 Rheology

Rheological tests are used to measure the deformation and flow behaviour of materials when an external force is applied, and to analyse their internal response to this applied stress. This tool allows quantitative measurements of hydrogel parameters, including viscosity, storage and loss moduli, all relevant characterization parameters. The rheological testing was carried out to understand how the varying concentrations of LAP, HAMA and PEGDMA in the hydrogel formulation influences the rheological properties of the hydrogels.

Several papers have studied the mechanical properties of the eye and its different components to better choose and design retinal scaffolds for transplantation. It was reported that the retina has a Young's modulus of approximately 20 kPa, with the photoreceptor layer having a modulus of 26 kPa [192], [193]. Furthermore, it was found that retinal scaffolds with a shear modulus lower than 35 kPa were difficult for surgical

handling and had a higher chance of failure during the transplantation process due to their fragility [193].

3.2.1 Background

Rheological experiments are conducted extensively in the published literature because of their significance in characterising hydrogels for tissue engineering. These tests provide an understanding of the hydrogel degree of crosslinking. They are also used to determine the optimal polymer concentration for the desired mechanical properties of the scaffold, depending on the targeted application [194]. Some papers are discussed below to understand the approaches that are used to study the rheological properties of hydrogels.

In Poldervaart *et al.* 2017, the authors used HAMA as a biomaterial for bone constructs, which meant that the rheological properties of the material were crucial to study [124]. The gel precursor solution was added at different concentrations to the rheometer setup and was cured in the rheometer using a UV light. The storage and loss moduli were recorded throughout the gelation using a constant strain of 1% and constant frequency of 1 Hz. It was found that the storage modulus is higher with increasing concentrations of HAMA.

Oudshoorn *et al.* (2007) fabricated methacrylated hyaluronic acid hydrogels with 5 degrees of methacrylation (DoM) and measured their rheological properties [146]. The storage and loss moduli were measured during *in situ* gel polymerisation in the rheometer, at constant 1% strain and 1 Hz frequency. During the gelation, the storage modulus increased with time while the loss modulus remained low throughout for all DoM. However, the values of the storage modulus were different depending on the DoM, with an order of magnitude difference between the lowest DoM (5.4%) and the highest (30%).

Noh *et al.* (2019) evaluated the use of hydrogels as bioink for 3D printing in tissue engineering [191]. They studied the rheological properties of their hydrogels by running a strain sweep (0.1 to 1000 Pa) and a frequency sweep (0.1 to 1000 Hz) and measuring the storage and loss moduli throughout the two experiments. Interestingly, their material had frequency dependent behaviour, where both the storage and loss moduli increased with increasing frequency and crossed over at about 80 Hz.

Velasco-Rodriguez *et al.* (2021) studied the mechanical properties of pure and hybrid GelMA-HAMA gels [190]. The gels were crosslinked in the rheometer and their storage and loss moduli were recorded. Additionally, they ran an oscillatory strain sweep followed by an oscillatory frequency sweep to measure the variation of storage moduli between the different gel formulations. They determined that the GelMA-HAMA hybrid gels exhibited higher storage modulus compared to the pure GelMA or HAMA hydrogels.

3.2.2 Materials and methods

The hydrogels characterised in this section were prepared using synthesised HAMA and PEGDMA, as described in Chapter 2. Lyophilized HAMA and PEGDMA were dissolved in 1X PBS containing 1% LAP to prepare the precursor solution. The LAP solution was first added to the PEGDMA and vortexed until the PEGDMA was fully dissolved. Following that, the new solution mixture was pipetted on top of the HAMA and left overnight at 4°C to dissolve the lyophilized HAMA.

The experiments were performed on a rheometer (MR 302 Anton Parr) equipped with a UV curing setup for *in situ* photopolymerization. This setup included a UV light (365 nm, 10 mW/cm², OmniCure S1500) shining through a clear bottom plate, and a flat 25 mm diameter parallel top plate. After the rheometer was calibrated, 105 µL of the gel precursor solution was pipetted onto the centre of the clear glass lower plate, and the top plate was lowered until a gap of 0.2 µm was achieved. A few drops of milliQ water were added around the edge of the sample to avoid material dehydration.

The protocol consisted of 3 experiments to get dynamic rheological measurements of the different material concentrations. At each point, the storage modulus G' and loss modulus G'' of the samples were collected

The first experiment was an oscillatory strain sweep that consisted of 60 points recorded over a range from 0.01% to 100%, at a constant frequency of 10 rad/s and at a room temperature of 25°C. The strain sweep had for aim to determine the linear viscoelastic region (LVR) of the material and chose a strain within that region to use in the test that followed. The hydrogel was cured *in situ* before this experiment was started. The LVR is the region where the storage modulus is independent of the applied strain [190].

The second experiment was an oscillatory frequency sweep that consisted of 45 points recorded over a range from 100 rad/s to 0.1 rad/s, at a constant strain of 0.1% (within the pre-determined LVR) and at a room temperature of 25°C. The frequency sweep had for aim to determine if the measured moduli displayed frequency dependent or independent behaviour. The hydrogel was cured *in situ* before the experiment was started.

The third experiment was an oscillatory time sweep, with *in situ* photopolymerization. It consisted of a ten-minute time sweep with UV light activation, at a constant strain of 0.1%, a constant frequency of 10 rad/s, and at a room temperature of 25°C. A data point was collected per second, giving a total of 600 points. There was a 1-minute standby before UV activation.

As there were three parameters to study for each formulation, two parameters were kept constant as the third was swept across five (or six in the case of LAP) different concentrations. The studied formulations are described in Table 3.7 below. All the experiments were performed in triplicate.

Table 3.7: Combinations of HAMA-PEGDMA-LAP concentrations chosen for the three rheology experiments. The first was to screen LAP concentrations, the second to screen PEGDMA concentrations and the last was to screen the HAMA concentrations.

	HAMA (%)	PEGDMA (%)	LAP (%)					
1	Constant	Constant	Variable					
	9	0	0.05	0.1	0.5	1	5	10
2	Constant	Variable	Constant					
	9	0 2 4 8 12	1					
3	Variable	Constant	Constant					
	3 6 9 12 15	4	1					

3.2.3 Results and discussion

For each experiment, the strain sweep, frequency sweep, and time sweep were plotted for the different polymer formulations. The storage and loss moduli values were plotted as the mean of three measurements with their standard deviation. All these plots can be seen in Figures 3.6, 3.7 and 3.8 below. Additionally, the storage modulus values from the time sweep experiments were plotted in a separate graph, without their standard deviations, to be able to directly compare them for the different gel formulations. Finally, the average storage modulus was calculated as the mean of the measured values, after the plateau was reached, for each gel formulation [124].

Figure 3.6: Stain, frequency, and time sweeps plotted for the different LAP concentrations with fixed PEGDMA and HAMA (n=3). The strain sweeps for all concentrations of LAP had a linear viscoelastic region extending to at least 1% strain. The frequency sweeps for all concentrations of LAP showed that the gels displayed frequency independent behaviour. The time sweeps for all concentrations of LAP showed an increase in the storage modulus until reaching a plateau.

Time Sweep

Figure 1 is a line graph showing the Storage and Loss Modulus of the Polyimide over time. The Y-axis is labeled 'Storage and Loss modulus (Pa)' and ranges from 1 to 100,000 on a logarithmic scale. The X-axis is labeled 'Time (secs)' and ranges from 0 to 700. The Storage Modulus (black line with circles) starts at approximately 10 Pa at 0 seconds, rises sharply to about 10,000 Pa by 100 seconds, and then remains stable at approximately 10,000 Pa until 700 seconds. The Loss Modulus (blue line with circles) starts at approximately 10 Pa at 0 seconds, rises to about 1,000 Pa by 100 seconds, and then remains stable at approximately 1,000 Pa until 700 seconds. Both moduli show a slight decrease after 200 seconds.

Time Sweep

Figure 1 is a line graph showing the Storage and Loss modulus of the epoxy resin system over time. The Y-axis represents Storage and Loss modulus in Pascals (Pa) on a logarithmic scale from 1 to 1,000,000. The X-axis represents Time in seconds (secs) from 0 to 700. The Storage modulus (black line with circles) starts at approximately 10,000 Pa at 0 seconds, rises sharply to about 1,000,000 Pa by 100 seconds, and remains stable thereafter. The Loss modulus (blue line with circles) starts at approximately 100 Pa at 0 seconds, peaks at about 1,000 Pa around 100 seconds, and then fluctuates between 10 and 1,000 Pa for the remainder of the test.

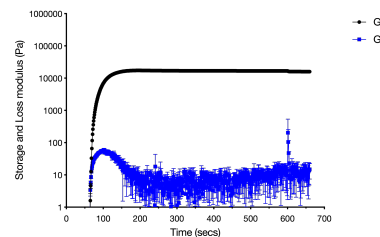
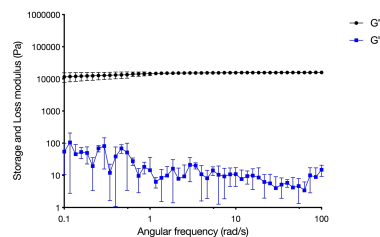
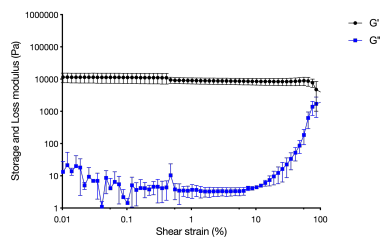
Figure 1 is a line graph showing the Storage and Loss modulus of the epoxy resin over time. The Y-axis is labeled 'Storage and Loss modulus (Pa)' and ranges from 1 to 100,000 on a logarithmic scale. The X-axis is labeled 'Time (secs)' and ranges from 0 to 700. The Storage modulus (black line with circles) increases rapidly from 10 Pa at 0 seconds to approximately 100,000 Pa by 100 seconds, then remains constant. The Loss modulus (blue line with circles) increases from 10 Pa at 0 seconds to approximately 1,000 Pa by 100 seconds, then remains constant with significant fluctuations between 100 Pa and 1,000 Pa.

Figure 3.7: Stain, frequency, and time sweeps plotted for the different PEGDMA concentrations with fixed LAP and HAMA ($n=3$). The strain sweeps for all concentrations of PEGDMA had a linear viscoelastic region extending to at least 1% strain. The frequency sweeps for all concentrations of PEGDMA showed that the gels displayed frequency independent behaviour. The time sweeps for all concentrations of PEGDMA showed an increase in the storage modulus until reaching a plateau.

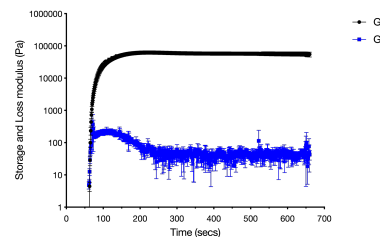
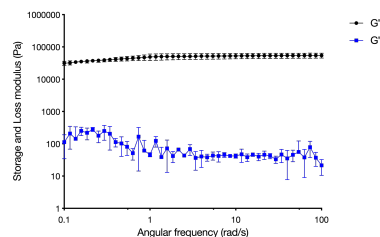
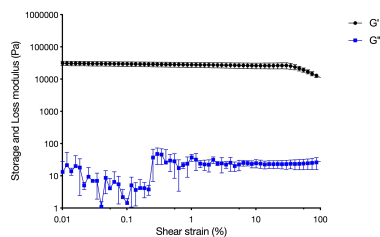
Figure 3.8: Stain, frequency, and time sweeps plotted for the different HAMA concentrations with fixed LAP and PEGDMA ($n=3$). The strain sweeps for all concentrations of HAMA had a linear viscoelastic region extending to at least 1% strain. The frequency sweeps for all concentrations of HAMA showed that the gels displayed frequency independent behaviour. The time sweeps for all concentrations of HAMA showed an increase in the storage modulus until reaching a plateau.

Time Sweep

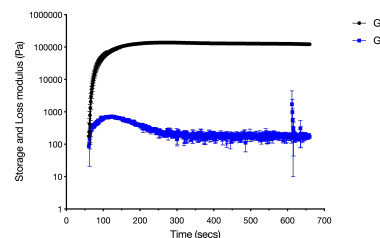
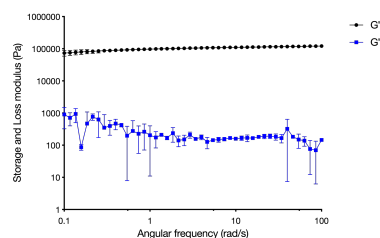
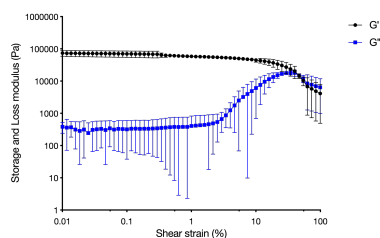
LAP 1%



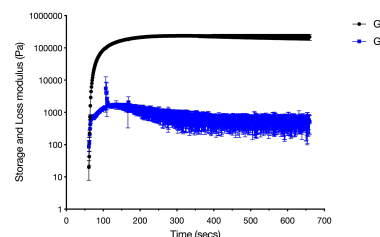
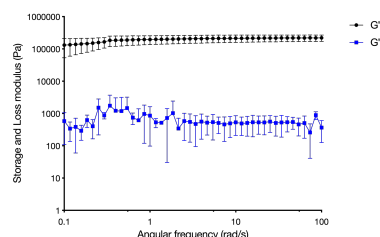
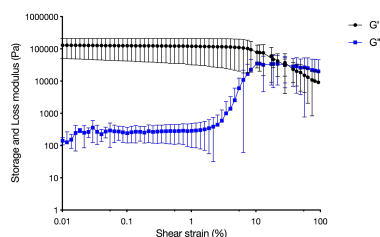
LAP 1%



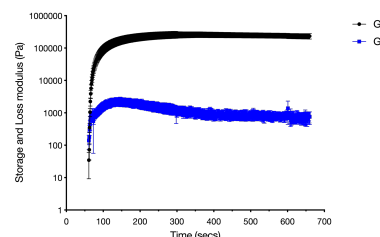
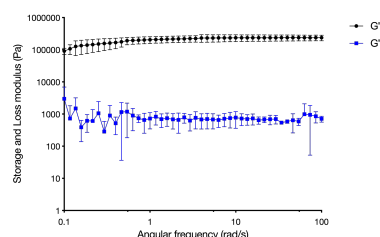
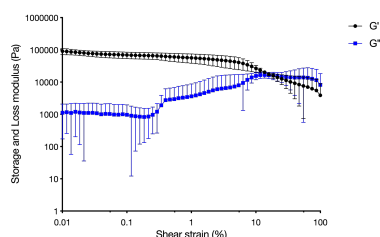
LAP 1%



LAP 1%



LAP 1%



3.2.3.1 Screening the LAP concentration

Figure 3.6 shows the strain, frequency and time sweeps for the various LAP concentrations with fixed PEGDMA and HAMA. The strain sweeps for all concentrations of LAP had a linear viscoelastic region extending to at least 1% strain. After 1% strain, G' began to decrease and G'' began to increase, until G' and G'' crossed over between 50% and 100% strain, meaning there was gradual breaking down of the gel microstructures. It was determined that 0.1% strain was within the LVR for all formulations and was chosen as the constant value for the frequency sweeps. Looking at the frequency sweeps for all LAP concentrations, the gels displayed frequency independent behaviour for all concentrations, and any frequency chosen within the range tested (0.1 to 100 Hz) could be chosen for the time sweep experiments.

The photo-crosslinking kinetics were studied by running an oscillatory time sweep for all concentrations of LAP as seen in Figure 3.6. The values of G' were replotted without their standard deviations in Figure 3.9 to better visualise and compare the rheological behaviour of the different hydrogel formulations. After the UV lamp was switched on, the storage modulus of all gel formulations increased and reached a plateau. For 0.05% LAP, the crosslinking rate was slower compared to the rest of the concentrations, and the plateau was only reached at approximately 400 seconds. The other concentrations reached a plateau at approximately 200 seconds.

The average storage modulus was calculated as the mean of the measured values, after the plateau was reached, for each gel formulation, and plotted in Figure 3.10 for the different concentrations of LAP. For the LAP concentrations ranging from 0.05% to 1%, the average storage modulus increased with increasing LAP concentration, however, above 1% LAP, it decreased. Therefore, LAP concentrations above 1% are most likely too high and affect the hydrogel mechanical properties. Ordinary one-way Anova with Tukey's multiple comparisons test were conducted on the values of G' and the results were significantly different for all the LAP concentrations.

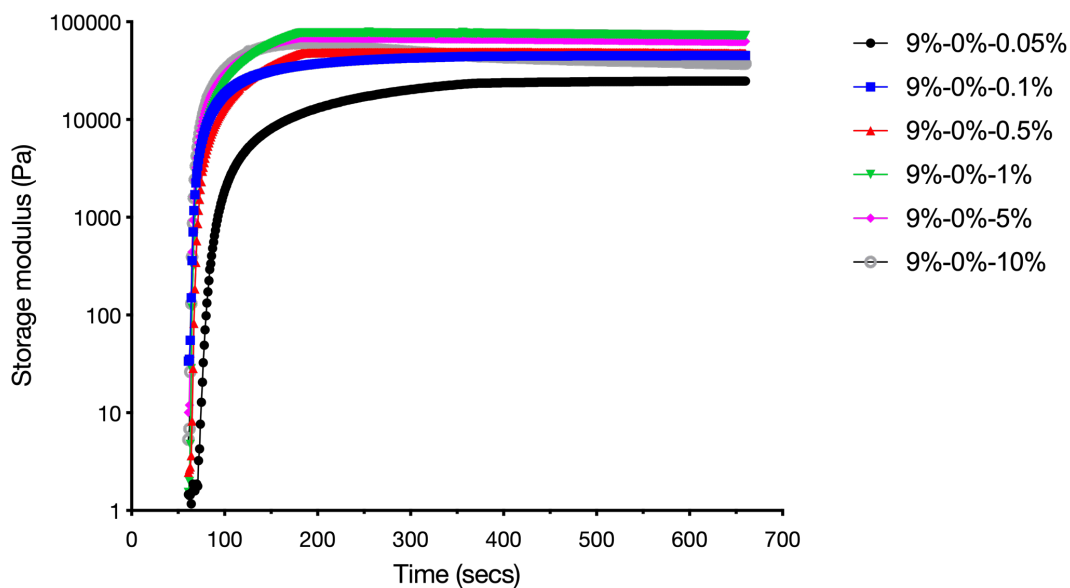


Figure 3.9: Oscillator storage modulus of the various LAP concentrations. Values were collected during *in situ* photopolymerization ($n=3$). After the UV lamp was switched on, the storage modulus of all gel formulations increased and reached a plateau. For 0.05% LAP, the crosslinking rate was slower compared to the rest of the concentrations, and the plateau was only reached at approximately 400 seconds. The other concentrations reached a plateau at approximately 200 seconds.

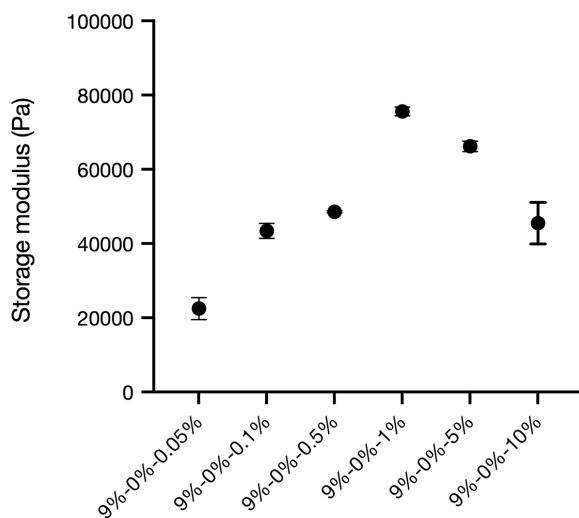


Figure 3.10: Average equilibrium storage modulus of the various LAP concentrations ($n=3$). For the LAP concentrations ranging from 0.05% to 1%, the average storage modulus increased with increasing LAP concentration, however, above 1% LAP, it decreased.

3.1.3.2 Screening the PEGDMA concentration

Figure 3.7 shows the strain, frequency and time sweeps for various PEGDMA concentrations with fixed LAP and HAMA. The strain sweeps for all concentrations of PEGDMA had a linear viscoelastic region extending to at least 1% strain. After 1% strain, G' began to decrease and G'' began to increase, until G' and G'' crossed over between 10% and 100% strain, meaning there was gradual breaking down of the gel microstructures. It was determined that 0.1% strain was within the LVR for all formulations and was chosen as the constant value for the frequency sweeps. Looking at the frequency sweeps for all PEG concentrations, the gels displayed frequency independent behaviour for all concentrations, and any frequency chosen within the range tested (0.1 to 100 Hz) could be chosen for the time sweep experiments.

The photo-crosslinking kinetics were studied by running an oscillatory time sweep for all concentrations of PEGDMA as seen in Figure 3.7. The values of G' were replotted without their standard deviations in Figure 3.11 to better visualise and compare the rheological behaviour of the different hydrogel formulations. After the UV lamp was switched on, the storage modulus of all gels increased and reached a plateau. For all concentrations of PEGDMA, the plateau was reached at approximately 200 seconds.

The average storage modulus was calculated as the mean of the measured values, after the plateau was reached, for each gel formulation, and plotted in Figure 3.12 for the different concentrations of PEGDMA. Looking at the graph, the storage modulus of the hydrogels increased with increasing PEGDMA concentration. These results were expected, as increasing the concentration of PEGDMA causes an increase in crosslinking density, which in turn causes an increase in storage modulus [124]. Ordinary one-way Anova with Tukey's multiple comparisons test were conducted on the values of G' and the results were significantly different for all the PEGDMA concentrations.

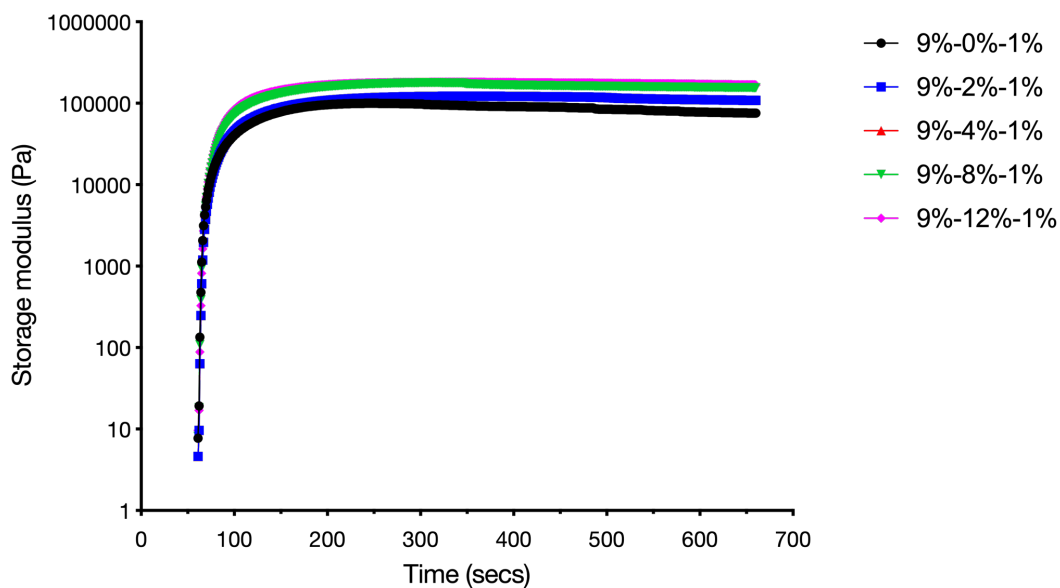


Figure 3.11: Oscillator storage modulus of the various PEGDMA concentrations. Values were collected during *in situ* photopolymerization ($n=3$). After the UV lamp was switched on, the storage modulus of all gels increased and reached a plateau. For all concentrations of PEGDMA, the plateau was reached at approximately 200 seconds.

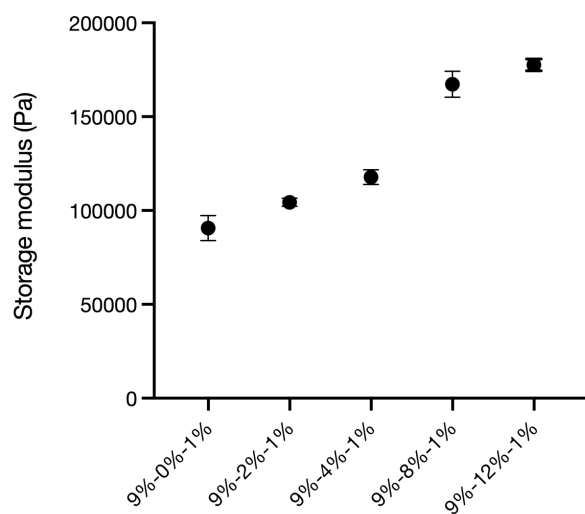


Figure 3.12: Average equilibrium storage modulus of the various PEGDMA concentrations ($n=3$). The storage modulus of the hydrogels increased with increasing PEGDMA concentration.

3.2.3.3 Screening the HAMA concentration

Figure 3.8 shows the strain, frequency and time sweeps for various HAMA concentrations with fixed LAP and PEGDMA. The strain sweeps for all concentrations of HAMA had a linear viscoelastic region extending to at least 1% strain. After 1% strain, G' began to decrease and G'' began to increase, until G' and G'' crossed over between 20% and 100% strain, meaning there was gradual breaking down of the gel microstructures. It was determined that 0.1% strain was within the LVR for all formulations and was chosen as the constant value for the frequency sweeps. Looking at the frequency sweeps for all HAMA concentrations, the gels displayed frequency independent behaviour for all concentrations, and any frequency chosen within the range tested (0.1 to 100 Hz) could be chosen for the time sweep experiments.

The photo-crosslinking kinetics were studied by running an oscillatory time sweep for all concentrations of HAMA as seen in Figure 3.8. The values of G' were replotted without their standard deviations in Figure 3.13 to better visualise and compare the rheological behaviour of the different hydrogel formulations. After the UV lamp was switched on, the storage modulus of all gels increased and reached a plateau. For all concentrations of HAMA, the plateau was reached at approximately 200 seconds.

The average storage modulus was calculated as the mean of the measured values, after the plateau was reached, for each gel formulation, and plotted in Figure 3.14 for the different concentrations of HAMA. Looking at the graph, the storage modulus of the hydrogels increased with increasing HAMA concentration. These results were expected, as increasing the concentration of HAMA causes an increase in crosslinking density, which in turn causes an increase in storage modulus [124]. Ordinary one-way Anova with Tukey's multiple comparisons test were conducted on the values of G' and the results were significantly different for all the HAMA concentrations.

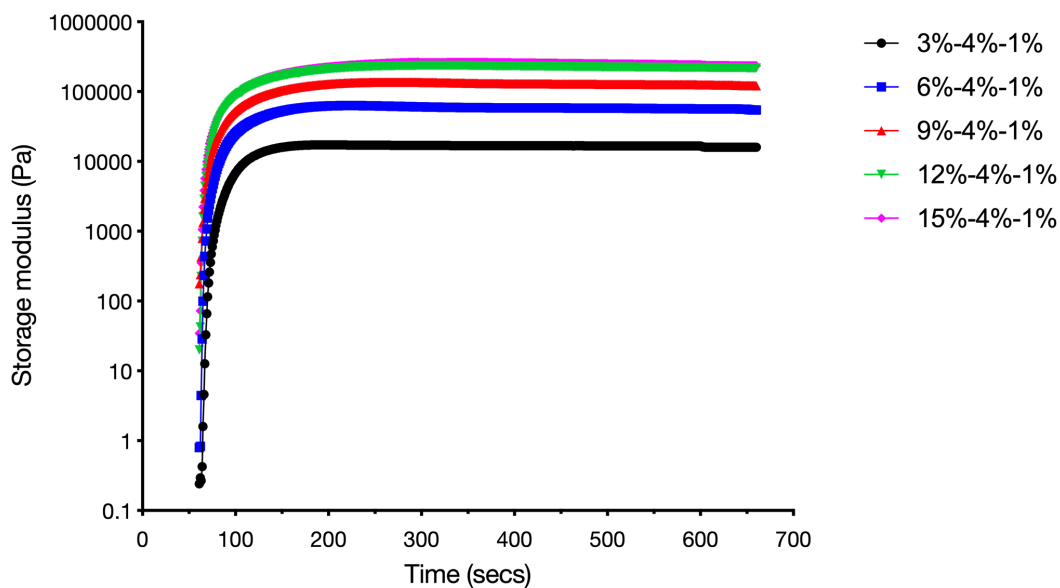


Figure 3.13: Oscillator storage modulus of the various HAMA concentrations. Values were collected during *in situ* photopolymerization ($n=3$). After the UV lamp was switched on, the storage modulus of all gels increased and reached a plateau. For all concentrations of HAMA, the plateau was reached at approximately 200 seconds.

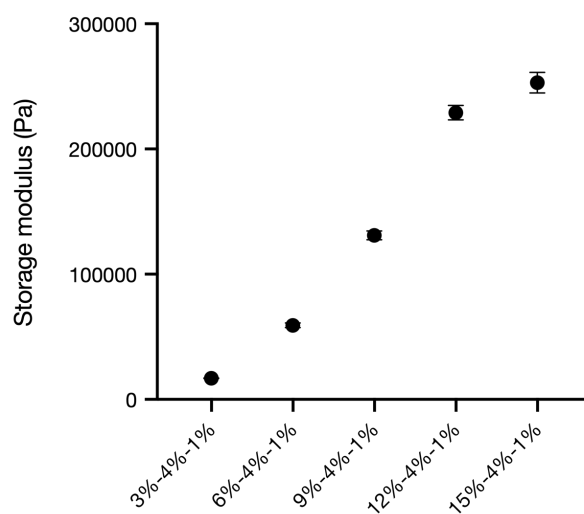


Figure 3.14: Average equilibrium storage modulus of the various HAMA concentrations ($n=3$). The storage modulus of the hydrogels increased with increasing HAMA concentration.

3.3 Diffusion

The aim of the patterned hydrogel scaffold is to be transplanted into the subretinal space of the host retina, located between the RPE layer and the photoreceptor layer. On one side of this space is the choroid, and on the other, the rest of the neural retina. Therefore, it is crucial that the material is porous enough to allow for small particles to diffuse across the hydrogel.

A pilot study was performed to study the diffusion of two dextran molecules in 5 selected hydrogel formulations *in vitro*. Dextran was used as a model molecule as it is a neutral glucan, that can be purchased with various molecular weights. It can also be easily conjugated with functional groups and dyes and is used in the literature for diffusion experiments to model proteins [195].

3.3.1 Background

Many tests have been conducted in the literature to determine how molecules diffuse across hydrogels and other materials, however the reported experiments differ in terms of experimental setup. Some papers are discussed below to understand the different approaches that are used to analyse particle diffusion across hydrogels.

In their 1993 paper, Lebrun *et al.* studied the diffusion of molecules through agar gel membranes [196]. They used sucrose to represent the smallest molecule sizes at 342 Da, and dextran at different molecular weights (10.5, 44, 74, 470 and 1950 kDa) to represent larger molecules. They placed their agar gel between two chambers in a diffusion vessel, as described in their group's previous publication and illustrated in Figure 3.15 below [197]. Each chamber had a volume of 150 cm³, could be temperature controlled, and contained a magnetic stirrer. The gel had an exposed diffusion area of 4.5 cm² and was sealed with an O-ring. One of the chambers was filled with either

sucrose or dextran in distilled water, while the second chamber was filled with plain distilled water.

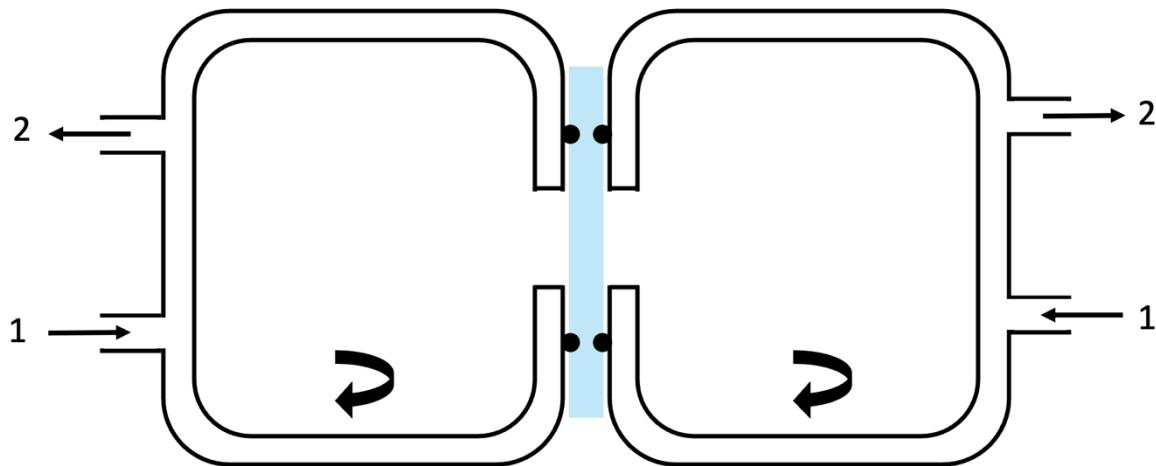


Figure 3.15: Diffusion vessel redrawn from [197]. (1) Water inlet; (2) Water outlet. The material was placed in between the two chambers and fixed with o-rings.

The experiment ran over the course of 24 hours, during which 0.4 cm^3 samples were collected from both chambers, with a total of 15 samples collected. The concentration of the molecules was measured using titration. They reported that for sucrose, the diffusivity in the agar gel was similar to that in water, however for the dextran molecules, the diffusion decreased with increasing molecular weight of dextran, with the largest 2 molecules unable to diffuse through certain membranes.

Cruise *et al.* (1998) studied the effect of PEGDA molecular weight and concentration on the diffusion of molecules [198]. They used a dual-chamber cell and placed the hydrogel between the two chambers, fixed with O-rings, and filled one chamber with a solution containing the molecules studied. Samples were collected at several time points, and

the molecule concentrations were measured using a UV-absorbance detector. They reported that for a fixed PEGDA molecular weight, increasing the polymer concentration led to a decrease in the diffusion of the molecules. Additionally, for a fixed PEGDA concentration, increasing the polymer molecular weight led to an increase in diffusion.

Nettles *et al.* (2004) studied the diffusion of 3, 70 and 500 kDa molecular weight dextran molecules, conjugated with fluorescein, from 1.5% (w/v) HAMA gels [140]. The hydrogels were fabricated, pre-swelled to equilibrium, and submerged in solutions of PBS containing the dextran molecules overnight at 4 °C to allow for the molecules to permeate the hydrogels fully. To quantify diffusion, the authors conducted fluorescence recovery after photobleaching experiments, a method commonly used to determine the kinetics of diffusion through biological samples [199]. They determined that the diffusion coefficients decreased with increasing dextran molecular weights.

Weber *et al.* (2008) studied the diffusion of proteins within their PEGDMA hydrogels as well as the effect of varying the PEG molecular weights on the diffusion behaviours of these proteins [200]. The 6 proteins they selected had molecular weights ranging from 5.7 kDa (Insulin) to 67 kDa (bovine serum albumin). The hydrogels were pre-swollen and incubated in a solution containing 1 mg/mL of the proteins studied for 24 hours at 4 °C. To quantify diffusion, they transferred the gels into fresh solutions of PBS at 37 °C after selected time points and measured the amount of protein released using a Micro-BCA™ Protein Assay Kit. They reported that the diffusion of the proteins decreased with increasing molecular weight. They also showed that for fixed protein, the diffusion rate decreased with decreasing PEG molecular weight.

Rizwan *et al.* (2017) studied the diffusion of glucose (180.16 Da) and bovine serum albumin (65 kDa) across a GelMA hydrogel [112]. They used a custom-made vessel, placed the hydrogel between two chambers, and filled one chamber with a solution containing the molecule studied. The diffusion of BSA was measured using a BCA assay, while the diffusion of glucose was measured using a glucose assay kit. The authors

reported that the glucose molecules diffused faster than the BSA molecules through the hydrogel.

3.3.2 Materials and methods

The five concentrations of HAMA and PEGDMA used in this chapter so far can be combined to form a total of 25 concentration formulations in total (the LAP concentration was fixed at 1%). These combinations can be found in Table 3.8. The pilot experiment consisted of the 4 concentration formulations at the corners of the table, as well as the concentration at the centre of the graph, 9% HAMA and 4% PEG, as that was the formulation that showed the most promising results thus far. The conditions chosen are highlighted in blue the table below.

Dextran molecules were used to represent the molecules that diffuse throughout the retina, as they are commercially available in different molecular weights, and can be labelled with fluorescent tags. For each of the 5 chosen formulations, hydrogels were fabricated with two molecular weights of dextran, 10 kDa and 70 kDa. These molecular weights were chosen based on the published literature, with the 70 kDa dextran modelling bovine serum albumin [200]. The dextran molecules were labelled with rhodamine B, a dye that emits a fluorescent signal with an emission peak at 590 nm when excited with a light at 570 nm. Additionally, negative control hydrogels with no dextran were fabricated. Experiments were performed in triplicate.

Table 3.8: Combinations of the HAMA and PEGDMA concentrations. The examined concentrations are highlighted in blue.

		HAMA				
		3%	6%	9%	12%	15%
PEGDMA	0%	3% - 0%	6% - 0%	9% - 0%	12% - 0%	15% - 0%
	2%	3% - 2%	6% - 2%	9% - 2%	12% - 2%	15% - 2%
	4%	3% - 4%	6% - 4%	9% - 4%	12% - 4%	15% - 4%
	8%	3% - 8%	6% - 8%	9% - 8%	12% - 8%	15% - 8%
	12%	3% - 12%	6% - 12%	9% - 12%	12% - 12%	15% - 12%

The hydrogels characterised in this section were prepared using the synthesised HAMA and PEGDMA, as described in Chapter 2. The gel precursor solution was prepared by weighing lyophilized HAMA, PEGDMA and LAP into separate Eppendorf tubes. The LAP was dissolved first with 1X PBS, and the two dextran molecules were added to the LAP in separate solutions at a concentration of 1 mg/mL. The LAP solutions were then added to the PEGDMA and vortexed until the PEGDMA was fully dissolved. Following that, the new solution mixtures were pipetted on top of the HAMA and left overnight at 4°C to dissolve the lyophilised HAMA. The tubes were kept covered in aluminium foil to prevent any polymerisation from the surrounding light as well as to prevent photobleaching of the rhodamine B.

The following day, 40 μ L of the precursor solutions was pipetted into 1 mL syringes, with the top already cut off, and crosslinked for 15 minutes under a 365 nm UV lamp. Once the polymerisation was completed, each hydrogel was incubated in 1 mL of PBS at 37 °C to begin the diffusion process. At each time point, the entire 1 mL was collected and stored in the fridge at 4°C and replaced with a fresh 1 mL of PBS. The following time

points were chosen to collect the incubated PBS: 10, 20 and 30 minutes, 1, 2, 4, 8, 12, 24 and 48 hours.

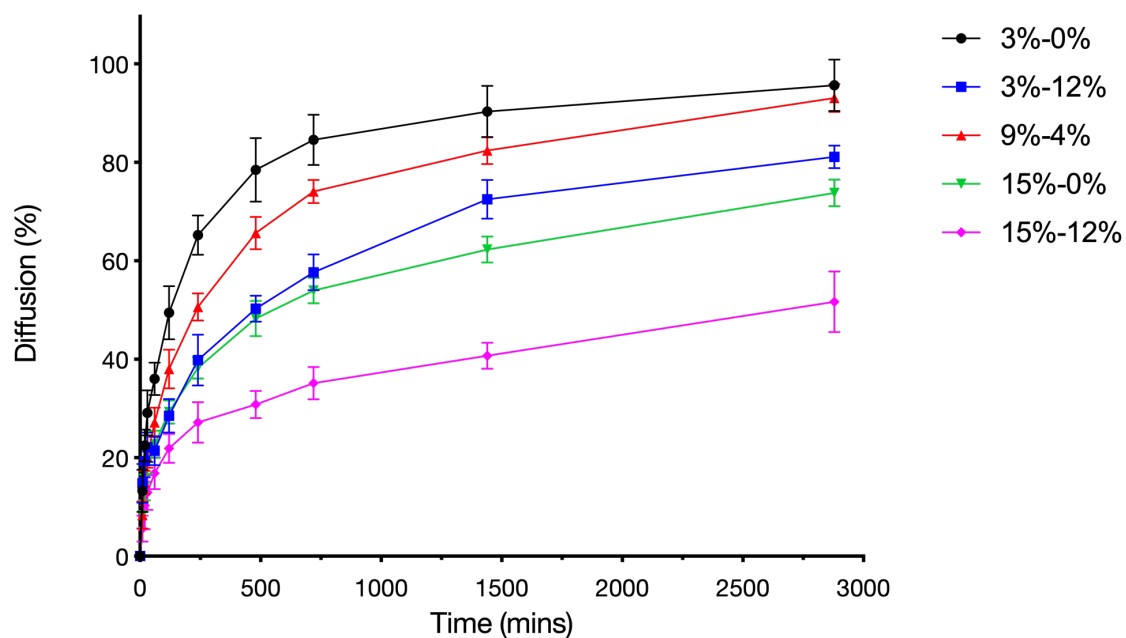
Additionally, a standard curve with known dextran concentrations was prepared to determine the mass of the dextran that diffused out of the hydrogel at each time point. The concentrations of dextran chosen for the standard curve were diluted in PBS and were the following: 1, 0.5, 0.1, 0.05, 0.01, 0.005 mg/mL, and a blank with just PBS. This was done for both molecular weights of dextran.

The samples were collected directly into 48 well plates at each time points and stored in the fridge until all samples were collected for analysis. The fluorescence of the rhodamine was measured on a Spectramax M5 plate reader (Molecular Devices) with an excitation wavelength of 550 nm and an emission wavelength of 575 nm.

3.3.3 Results and discussion

The standard curve was drawn for the two dextran molecules, with a best-fit linear equation, to extract the equation that relates the fluorescence intensity measurements from the plate reader to the known dextran concentrations. Then, for each of the three replicates per condition, the average fluorescence intensity was calculated by subtracting the average blank intensity from the average measured fluorescence intensity for each of the corresponding gel conditions. The average fluorescence intensity was then converted to concentration (and subsequently mass) using the linear equation. Finally, the percentage of dextran release was calculated by dividing the diffused mass of dextran by the initial mass of dextran added to the hydrogels. The cumulative diffusion percentage was plotted over time for the 70 kDa and 10 kDa dextran molecules and can be seen in Figure 3.16.

A



B

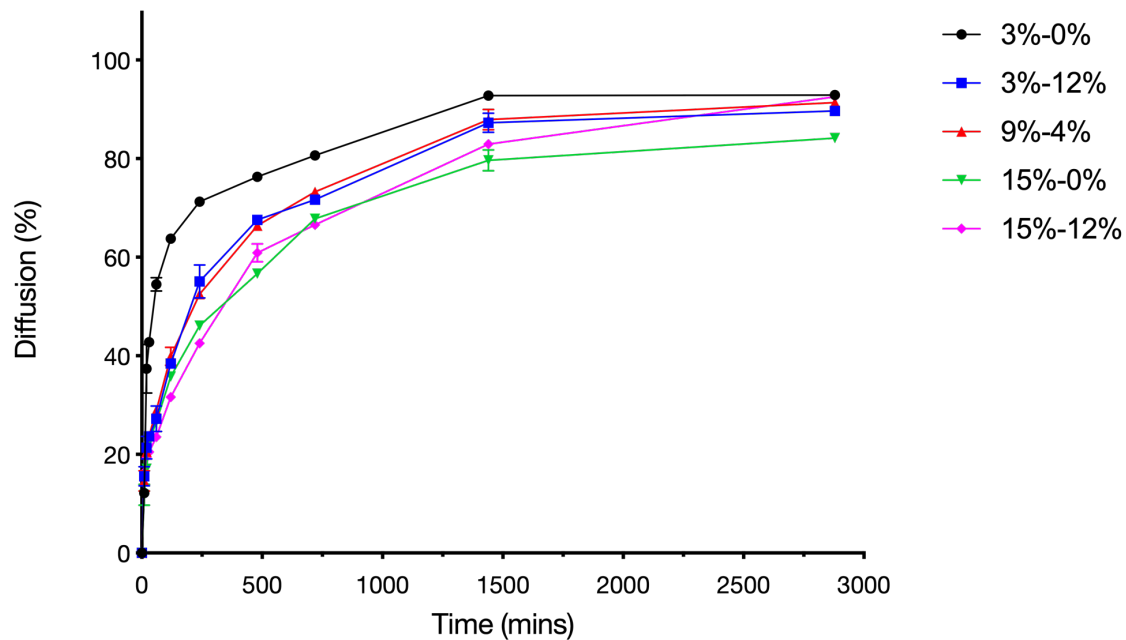


Figure 3.16: Cumulative dextran diffusion percentages. The values were plotted for the 5 hydrogel conditions over time for the 70 kDa (A) and 10 kDa (B) molecular weights of dextran ($n=3$).

Looking at the first graph, which corresponds to the 70 kDa dextran molecules, the plots of the cumulative diffusion percentages were different for the various hydrogel conditions tested, in terms of the rates at which dextran was released, and the total amount of dextran released.

The 3% HAMA – 0% PEGDMA hydrogels had the highest percentages of dextran released over the course of the experiment, and the fastest release rate. This data fits with the knowledge that those gels had the least amount of polymer in the network compared to the rest of the conditions, and thus a lower crosslinking density. As the porosity of hydrogels is inversely related to the crosslinking density [124], [141], these gels had the largest pores. The 15% HAMA – 12% PEGDMA hydrogels had the lowest percentages of dextran released over the course of the experiment, and the slowest release rate. These gels had the highest amount of polymer in the network and the highest crosslinking density, which meant that they had the smallest pores. Thus, the dextran could not diffuse out of the hydrogels as easily and as quickly as the other hydrogel concentrations. Directly comparing the highest and lowest polymer concentrations, the 3% HAMA – 0% PEGDMA hydrogels had approximately 96% of the 70 kDa dextran diffuse out of them, while the 15% HAMA – 12% PEGDMA had approximately 52%, lower by two-folds.

Comparing the other hydrogel concentrations with one fixed parameter, the trends were similar. Fixing the HAMA concentrations (either 3% or 15%), the increase in PEGDMA concentration from 0% to 12% lead to a decrease in the rate and the total amount of dextran diffused out of the hydrogels. Fixing the PEGDMA concentrations (either 0% or 12%), the increase in HAMA concentration from 3% to 15% also lead to a decrease in the rate and the total amount of dextran diffused out of the hydrogels. All these results were due to the increase in crosslinking density and decrease in porosity of the hydrogels when one of the polymer concentrations is increased. The 9% HAMA – 4% PEGDMA could not be as easily compared to the rest of the concentrations, however when compared to the 3% HAMA – 0% PEGDMA, it could be seen that, even though the rate

of diffusion was lower, the total amount of 70 kDa dextran diffused was almost the same after 48 hours. Taking into consideration Tukey's multiple comparison test, the diffusion behaviours of all the concentrations were significantly different when compared two at a time (except between 3% – 12% and 15% – 0%).

Looking at the data from the 10 kDa dextran diffusion experiments, the differences between the polymer concentrations was not as apparent, except for the 3% HAMA – 0% PEGDMA, which had a higher diffusion rate when compared to the other hydrogels. The rest of the polymer concentrations had similar rates. Looking at the final cumulative diffusion percentages, they all lied between 84 and 93%. Taking into consideration Tukey's multiple comparison test), the diffusion behaviours of all the concentrations were significantly different when compared two at a time (except between 3% – 12% and 15% – 0%).

To better compare the behaviour of the two dextran molecules, one graph was plotted per gel formulation, and contained the data for both dextran molecules. Looking at Figure 3.17, the 3% HAMA – 0% PEGDMA hydrogels had no significant difference in diffusion between the 70 kDa and the 10 kDa dextran molecules. When one of the concentrations was increased and the other was kept fixed, increasing the size of the dextran molecule led to a decrease in the rate and the total amount of dextran diffused out of the hydrogels. When both concentrations of the polymers were increased, increasing the size of the dextran molecule led to an even larger decrease in the rate and the total amount of dextran diffused out of the hydrogels. This was due to the decrease in the hydrogel pore sizes with increasing polymer concentration.

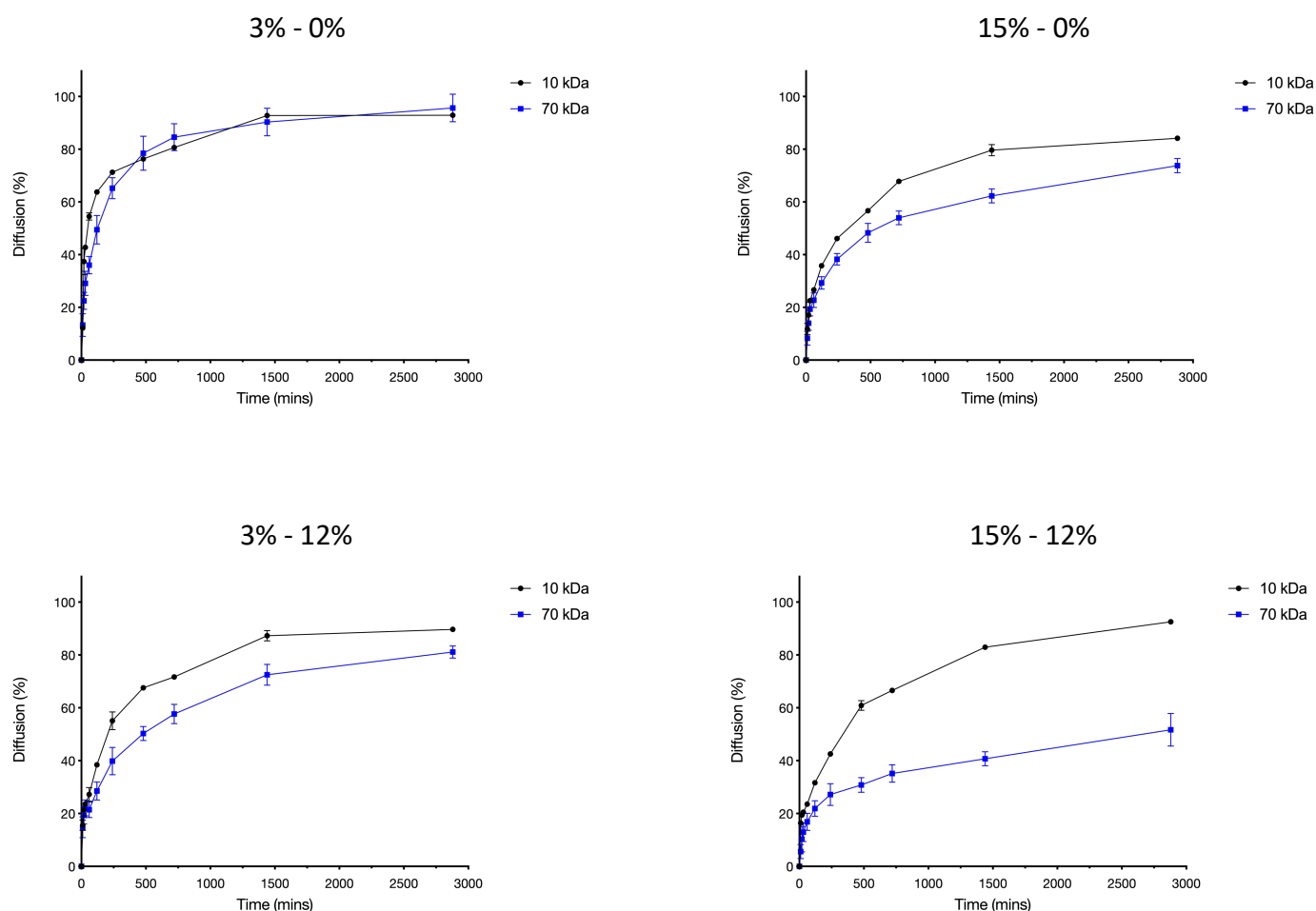


Figure 3.17: Cumulative dextran diffusion percentages plotted for 4 of the 5 hydrogel conditions. Values were plotted over time for the 70 kDa and 10 kDa molecular weights of dextran (n=3).

Finally, the plot of the 9% HAMA – 4% PEGDMA hydrogels, as seen in Figure 3.18, shows that there was no significant difference in diffusion between the 70 kDa and the 10 kDa dextran molecules. This hydrogel formulation was the one that showed the most promising results thus far, and this experiment only further confirmed this.

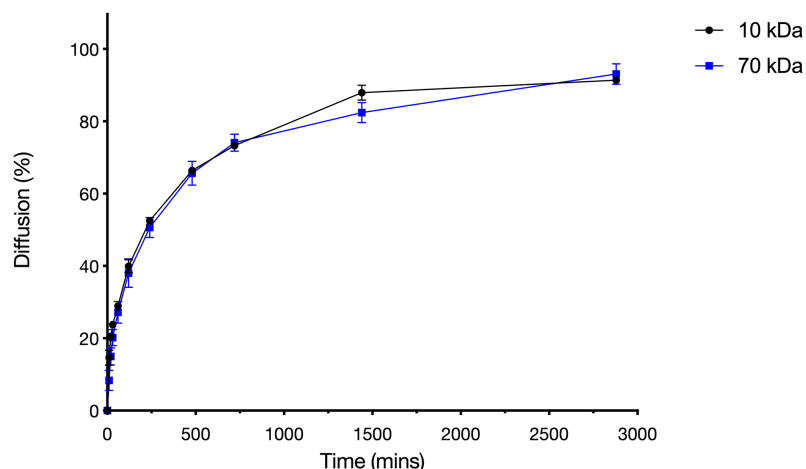


Figure 3.18: Cumulative dextran diffusion percentages plotted for the 9% HAMA – 4% PEGDMA hydrogel formulation. Values were plotted over time for the 70 kDa and 10 kDa molecular weights of dextran (n=3).

3.4 Degradation:

The patterned hydrogel scaffold is intended to be transplanted *in vivo* into the subretinal space with photoreceptors cultured in the channels, with the aim of integrating those cells into the host retina and forming synaptic connections with the host bipolar cells. There is no literature outlining the timeline required for the transplanted photoreceptor cells to form these connections, therefore the exact amount time the transplanted scaffold is required to remain in the host retina is unclear. As the required degradation profile is unknown, HAMA – PEGDMA hydrogels with varying concentrations of both polymers were tested, to study their degradation profile when incubated with hyaluronidase, and to produce a library of different hydrogels with varying degradation rates.

Hyaluronidase is an enzyme that catalyses the degradation of hyaluronic acid in the body. It is present in many organs, including the eye, liver, kidneys, and in body fluids, including the blood and tears [201]. This enzyme breaks down hyaluronic acid into

monosaccharides by cleaving the glycosidic bond, as shown in the degradation reaction in Figure 3.19.

Looking at the degradation mechanism more closely, the amino acid within the enzyme that is involved in the degradation is Glutamic acid (Glu). This amino acid is located at the active site of the enzyme, and is responsible for the catalysed reaction, making it the catalytic residue of the enzyme and the proton donor for the reaction [202], [203]. The other component involved in the reaction is the carbonyl group of the N-acetyl-D-glucosamine disaccharide in the HA chain, which serves as the nucleophile [135], [204].

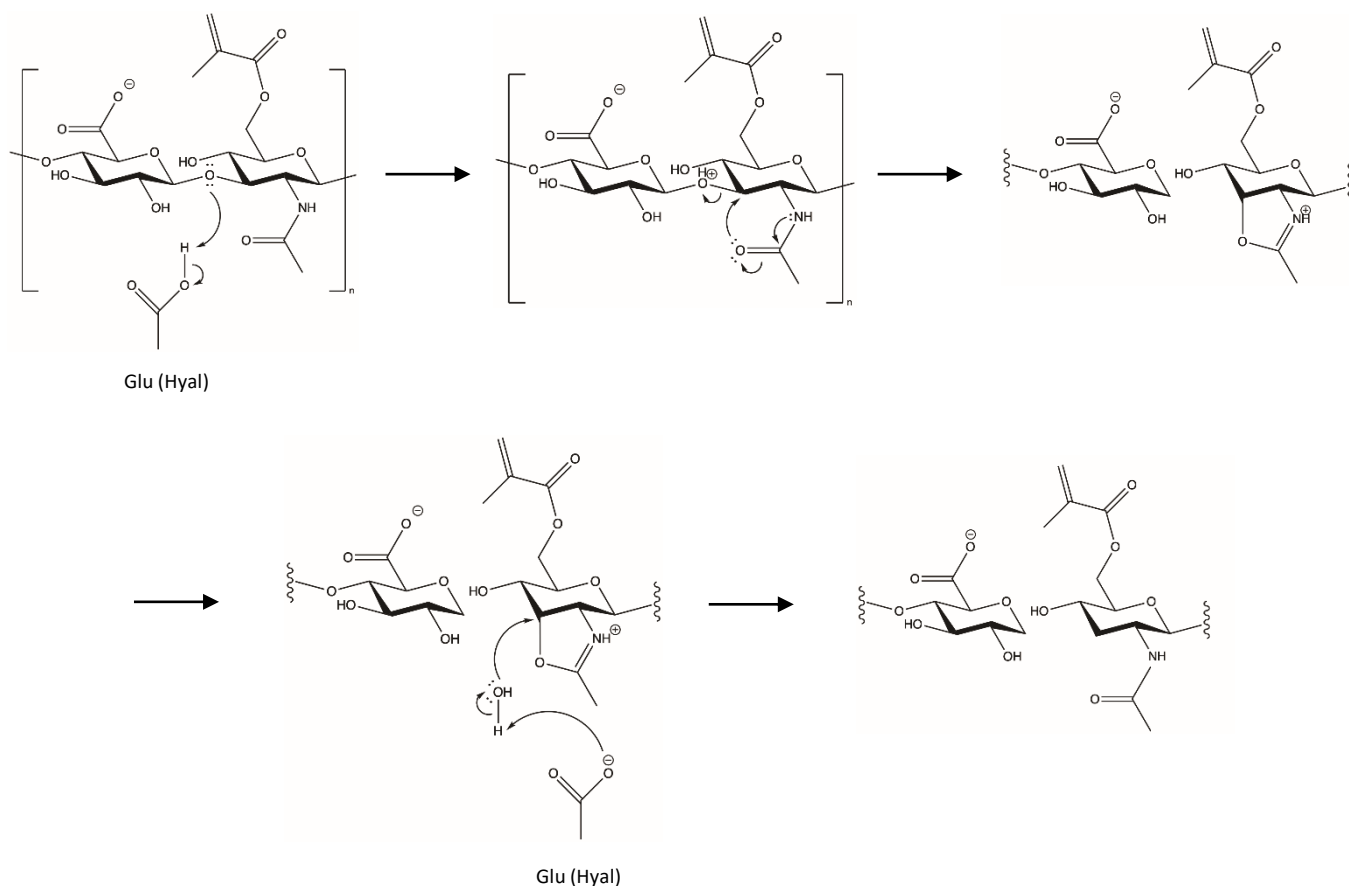


Figure 3.19: Mechanism of hyaluronidase degradation of HAMA. Figure redrawn from [135].

The PEGDMA bonds within the hydrogels do not degrade through the action of hyaluronidase. Instead, they degrade slowly through ester hydrolysis leaving the 6kDa PEG chains as metabolic waste to be cleared from the eye through the choroidal bloodstream, and subsequently, by the kidneys [205]. The kidneys are capable of clearing solutes with a molecular weight cut-off of 30 to 50 kDa [206], meaning the 6kDa PEG waste can be easily cleared out of the body following degradation.

3.4.1 Background

Degradation experiments are conducted extensively in the published literature because of their importance in characterising hydrogels for tissue engineering. Some papers are discussed below to understand the different approaches that are used to measure degradation.

Poldervaart *et al.*'s 2017 paper used HAMA as a biomaterial in bone regenerative medicine. The hydrogels were polymerised, and their weight was directly measured at time 0 (W_0) [124]. Following that, they were submerged in PBS containing 2.6 U/mL of hyaluronidase, and removed at multiple time points, lyophilized, and weighed to get their dry weight (W_{dry}). The experiment was conducted for a total of three weeks. The degradation was calculated by dividing the dry weight at specific time points by the dry weight at time 0:

$$Degradation (\%) = \frac{W_{dry}}{W_0} \times 100\%$$

The hydrogels degraded the fastest in the lower concentration gels, with the lowest concentrations degrading after just 24 hours.

Masters *et al.* (2005) studied the effect of adding PEGDA to HAMA in terms of degradation rates [141]. The hydrogels were prepared by mixing 2% HAMA with 0, 5 and 10% PEGDA. The hydrogels were polymerised and submerged in PBS containing 5 U/mL of hyaluronidase. The hydrogels containing only HAMA degraded after 48 hours,

significantly faster than those containing PEGDA. The gels containing 5% PEGDA degraded after 285 hours, while those containing 10% PEGDA only lost 10% of their mass after 300 hours. Therefore, it could be concluded that incorporating PEG into the HAMA hydrogel slows down degradation considerably and that PEG is a suitable polymer for controlling the degradation rates of the hydrogels.

Velasco-Rodriguez *et al.* (2021) studied the degradation of hybrid GelMA – HAMA hydrogels, as well as gels composed of the individual polymers, all at different concentrations [190]. The hydrogels were fabricated and weighed at time point 0, and then incubated in a solution of 3 unit/mL of collagenase type II to degrade the gels. As the enzyme activity depletes over time, they replaced the solution every 4 hours to ensure continuous degradation. Every 2 hours, the hydrogels were removed, washed, lyophilised, and weighed to determine the dry weight at specific time points. The experiment was conducted for 24 hours. The degradation was calculated using the following formula:

$$\text{Degradability (\%)} = \frac{W_0 - W_{dry}}{W_0} \times 100\%$$

Since the enzyme used was collagenase, the pure GelMA scaffolds were completely degraded by the end of the experiment, which the authors hypothesised could have been due to the continuous replacement of the enzyme solution. In contrast, the HAMA hydrogels were barely degraded during the experiment as they could not be degraded by collagenase (which was predicted). The hybrid GelMA – HAMA hydrogels were degraded in different amounts, depending on their composition. For a fixed HAMA concentration, the degradation percentage increased with increasing GelMA percentage, and for a fixed GelMA concentration, the degradation percentage decreased with increasing HAMA concentration. They concluded that hydrogels with higher polymer concentrations had stronger covalent bonds, decreasing the effect of the enzyme on the hydrogel.

Rizwan *et al.* (2017) studied the degradation of 8 mm 30 % GelMA disks over 7 days in collagenase [112]. The hydrogels were incubated in solutions of 1, 10 and 20 unit/mL of collagenase, to evaluate the effect of increasing the enzyme concentration on the hydrogel degradation rate. The enzyme solution was changed daily to maintain the enzyme activity over the course of the experiment. Samples from the solution were collected at regular time intervals, and the degradation was measured by running a BCA assay on the samples collected to measure the total amount of protein released into the solution due to degradation. They reported that increasing the concentration of collagenase increased the degradation of the hydrogels, with the hydrogels incubated in the 10 and 20 unit/mL collagenase degrading completely after 24 hours, and those in 1 unit/mL degrading by 55% by the end of the 7 days.

Khademhosseini *et al.* (2006) assessed the degradation of HAMA hydrogels with encapsulated cells at 37 °C in a 10,000 unit/mL hyaluronidase solution in DMEM media [145]. They monitored the degradation by taking microscopy images of the hydrogels at times 0, 1.5 and 25 hours, and could visually see the degradation of the hydrogels, but did not produce any quantitative measurements of degradation.

Burdick *et al.* (2005) studied the degradation of 9 mm diameter HAMA hydrogels incubated at 37 °C with 100 unit/mL of hyaluronidase and recorded the amount of time it took for the hydrogels to be fully degraded [149]. The solution was collected and replaced every 48 hours and stored at -20 °C until all samples were collected for analysis. Several HAMA concentrations and molecular weights were tested to study the effect of varying these parameters on the degradation. More analysis was conducted by measuring the amount of uronic acid (a degradation component of hyaluronic acid) present in the degradation solutions using an established protocol (carbazole reaction) published by Bitter and Muir's 1962 paper. In terms of the total degradation time, they reported that for a fixed molecular weight of HA, the number of days needed to achieve full degradation increased with increasing polymer concentration, and that for a fixed concentration, the number of days to full degradation decreased with increasing

molecular weight. In terms of the uronic acid measurements, the results showed similar trends, with the hydrogel degradation rate increasing with decreasing HA molecular weight for a fixed concentration.

Sahoo *et al.* (2008) used the same method used in Burdick 2005 to measure degradation [207]. The amount of uronic acid in the degradation media was measured, using the methods in Bitter and Muir' 1962 paper, for HAMA hydrogels at different concentrations. The percentage of uronic acid released increased with decreasing polymer concentration.

Seidlits *et al.* (2010) studied the degradation of HAMA hydrogels, with various degrees of functionalisation, incubated in a solution of 50 unit/mL of hyaluronidase at 37 °C [143]. The initial weight of the hydrogels, pre-swollen for 24 hours at 4 °C, was recorded. During the degradation experiment, the weights of the hydrogels were recorded at specific time points and normalised to the initial weight. The hydrogel degradation varied with the different hydrogel degrees of modifications in an inverse relationship, with the highest degrees of modification degrading the slowest (24 hours) and the lowest degrading the fastest (6 hours).

3.4.2 Pilot study

As mentioned in section 3.3, five concentrations of HAMA and PEGDMA were combined, with a fixed LAP concentration of 1%, to form a total of 25 combinations of concentrations in total. These combinations can be found in Table 3.9 below.

As the planned degradation experiment required a month of collecting data as well as 25 different parameter combinations, a pilot study was completed first to optimize the experiment. The pilot experiment consisted of the 4 concentration combinations at the corners of the table shown below (highlighted in blue), with the assumption that the 3%

- 0% would degrade the fastest, as it contained the lowest quantity of polymers, while the 15% - 12% would degrade slowest, having the highest quantity of polymer.

Table 3.9: Possible combinations of the HAMA and PEGDMA concentrations. Examined concentrations are highlighted in blue.

		HAMA				
		3%	6%	9%	12%	15%
PEG	0%	3% - 0%	6% - 0%	9% - 0%	12% - 0%	15% - 0%
	2%	3% - 2%	6% - 2%	9% - 2%	12% - 2%	15% - 2%
	4%	3% - 4%	6% - 4%	9% - 4%	12% - 4%	15% - 4%
	8%	3% - 8%	6% - 8%	9% - 8%	12% - 8%	15% - 8%
	12%	3% - 12%	6% - 12%	9% - 12%	12% - 12%	15% - 12%

3.4.2.1 Materials and methods

The hydrogels in this characterization experiment were prepared using the synthesised HAMA and PEGDMA, as described in Chapter 2. The gel precursor solution was prepared by weighing lyophilized HAMA, PEGDMA and LAP into separate Eppendorf tubes. The LAP was dissolved first with 1X PBS and then added to the PEG and vortexed until the PEGDMA was fully dissolved. Following that, the new solution mixtures were pipetted on top of the HAMA and left overnight at 4°C to dissolve the lyophilised HAMA. The tubes were kept covered in aluminium foil to prevent any polymerisation from the surrounding light.

The following day, 40 µL of the precursor solution was pipetted into 1 mL syringes, with the top already cut off, and crosslinked for 15 minutes under a 365 nm UV lamp. Once the polymerisation was completed, each hydrogel was placed in a well of a 48 well plate

and incubated in 1 mL of PBS overnight to pre-swell the hydrogels and wash away any unbound materials. Experiments were run in triplicate.

The next day, the PBS was aspirated and replaced with a fresh solution of hyaluronidase at a concentration of 22 units/mL. The hyaluronidase solution was prepared by dissolving hyaluronidase powder (539 units/mg, purchased from Sigma Aldrich) in a hyaluronidase buffer solution, as described in the specification sheet of the enzyme (0.02 M phosphate buffer at pH 7, 77 mM of sodium chloride, and 0.01% BSA at 1 mg/mL). The concentration of hyaluronidase was chosen based on a published paper reporting the concentration of hyaluronidase in the vitreous, to replicate the *in vivo* scenario as closely as possible [208].

At each time point, the entire 1 mL was collected and stored in the fridge at 4°C and replaced with a fresh 1 mL of the hyaluronidase solution. This experiment was run for 5 days. The enzyme solution was changed daily to maintain the enzyme activity over the course of the experiment, similarly to Rizwan 2017 [112].

The degradation was quantified by running a Micro-BCA™ Protein Assay Kit (Thermo Fisher) to measure the amount of protein released in the solution, also similarly to Rizwan 2017 [112]. This method was chosen instead of the weight difference measurements, used in Seidlits 2010 and Velasco-Rodriguez 2021 [143], [190], due to the inaccuracies that can occur during the weighing process. Poldervaart 2017's method of freeze drying the hydrogel to compare the dry weights was more accurate, however was not chosen as it doubles the duration of the experiment [124].

The Micro-BCA™ Protein Assay Kit is used to quantify total protein in a given solution by colorimetric detection. It uses bicinchoninic acid (BCA) as the detection reagent for Cu^{+1} in the solution, formed when Cu^{+2} is reduced by a protein in an alkaline solution. When two molecules of BCA bind to one molecule of Cu^{+1} , the reaction forms a purple-coloured product, that exhibits a strong absorbance at 562 nm, and that increases

linearly with increasing concentrations. The reduction of Cu^{+2} is caused by several factors, including the protein structure and composition, and the presence of four amino acids (cysteine, cystine, tryptophan and tyrosine). In the case of the HAMA, the reduction is caused by a combination of contributions from the amine and the carboxylic acids, circled in Figure 3.20 below. The kit does not react with anything on the PEGDMA chain, meaning that any colorimetric reaction observed is solely from the HAMA degradation products released into the solution during the experiment.

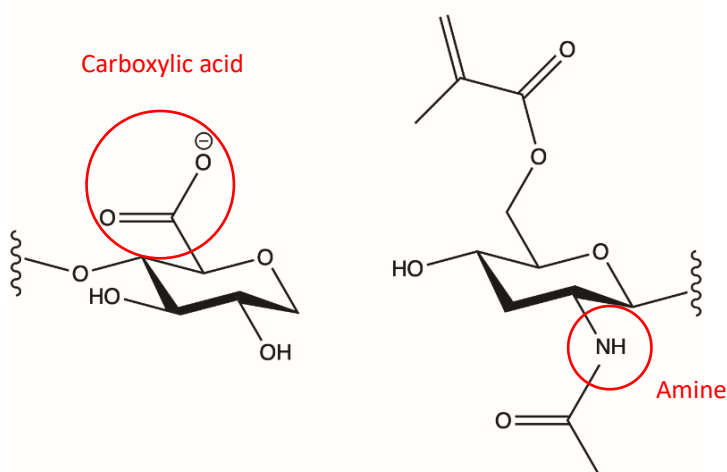


Figure 3.20: Chemical structures of the HAMA degradation products.

The collected samples for the 5-day experiment were pipetted into a 384 well plate (20 μL per well) in triplicate for each hydrogel, in addition to the standard curve prepared with known concentrations of BSA, as specified in the kit. Additionally, hyaluronidase controls were also pipetted to account for any background signal. An equal volume of the working reagent was added to the wells, and a clear-green colour was directly observed. The plate was placed on a shaker for 30 seconds, centrifuged at 100 x g for 2 minutes, and then incubated at 37 °C for 2 hours. The plate was cooled to room

temperature before measuring the absorbance at 562 nm using a plate reader (EnVision Multimode plate reader, Perkin Elmer).

3.4.2.2 Results and discussion

The standard curve was drawn, with a best-fit polynomial equation, to extract the equation that relates the absorbance intensity measurements from the plate reader to the known BSA concentrations. Then, for each of the three replicates per condition, the average absorbance intensity was calculated by subtracting the corresponding average hyaluronidase control intensity from the average measured absorbance intensity for each of the corresponding gel conditions.

The average absorbance intensity was then converted to concentration (and subsequently mass) using the polynomial equation. Finally, the percentage of degradation was calculated by dividing the mass of the released degradation products by the initial mass of HAMA in the hydrogels, according to the concentration. The cumulative degradation percentage was plotted over time and can be seen in Figure 3.21.

Looking at the graph, the 3% HAMA – 0% PEGDMA hydrogels had the highest percentage of degradation over the course of the experiment, reaching 24% degradation after 5 days. This data fits with the knowledge that those gels had the least amount of polymer in the network compared to the rest of the conditions, and thus a lower crosslinking density, and largest pores, allowing the enzyme to seep more easily into the hydrogel, further degrading the crosslinks and increasing the amount of degradation products released into the solution [149], [190].

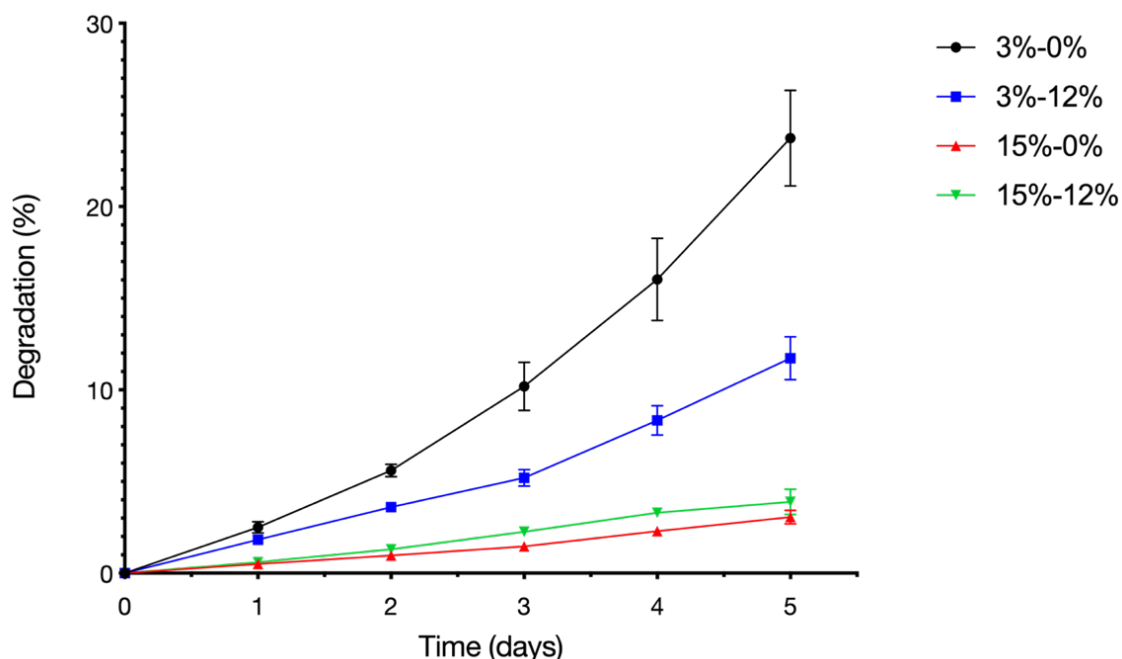


Figure 3.21: Cumulative degradation percentage of the 4 concentration conditions. The data was collected over the course of 5 days (N=3, n=3).

Fixing the HAMA concentration at 3% and increasing the PEGDMA concentration from 0% to 12% lead to a lower degradation rate and total degradation percentage after 5 days, reaching a total of 12% degradation. These gels had the same amount of HAMA, but the addition of the PEGDMA increased the crosslinking density of the gels, as well as strengthened the bonds, slowing down the effect of the enzyme, and slowing down the total degradation [149], [190].

Both hydrogel conditions containing 15% HAMA had similar degradation rates and total degradation percentage after 5 days. Compared to the 3% HAMA, these hydrogels degraded much slower, and only reached a total of 3-4%. Increasing the concentration of HAMA also increased the crosslinking density, which decreased the effect of the enzyme and the amount of degradation products. However, unlike the lower HAMA

concentrations, the increased concentration of PEGDMA did not have as much of an effect on the degradation [149], [190].

Statistical analysis on the data (2-way Anova with Tukey's multiple comparison test) reveals that all the plot comparisons, except one, were significantly different. The only non-significant comparison was between the 15% HAMA – 0% PEGDMA and the 15% HAMA – 12% PEGDMA hydrogels.

The results of the pilot experiment demonstrated that the degradation rate and the total amount of degradation could be controlled depending on the concentration of both HAMA and PEGDMA, to a certain extent. However, after 5 days, none of the hydrogels degraded fully. A longer experiment was required to study the long-term effect of hyaluronidase on the hydrogels.

3.4.3 Full study

The full experiment included all 25 combinations of HAMA and PEGDMA concentrations as seen in Table 3.9. The experiment was run for a total of 30 days, using the same protocol used in the pilot, with some minor modifications.

3.4.3.1 Materials and methods

The hydrogels in this characterization experiment were prepared using the same methods described in 3.4.2.1. Triplicate hydrogels were fabricated for each of the 25 conditions, placed into the wells of 48-well plates, and incubated with the hyaluronidase solution. Additionally, 5 conditions were chosen as negative controls, and incubated with the buffer solution. Those conditions included the 4 used in the pilot experiment, as well as the 9% HAMA – 4% PEGDMA hydrogel condition. Unlike the pilot study where the hyaluronidase controls were added only to the 384 well plate for the final measurements, both the hyaluronidase and the plain buffer solutions were added to the 48 well plate

ensure that they are incubated with the hydrogels under the same conditions, and the degradation media from the hydrogels for a given day were normalised to the corresponding controls.

All solutions were collected daily and placed in the freezer at -20 °C to store until the measurements were performed. These included the hyaluronidase and plain buffer solutions incubated with and without the hydrogels. After 30 days, the experiment was completed, and all of the collected samples were thawed in the fridge overnight. These samples were similarly pipetted into 384 well plates, and one plate was used for samples collected on the same day. A standard curve was also added per plate.

3.4.3.2 Results and discussion

The degradation graphs were plotted using the same approach as described in section 3.4.2.2. Graphs were grouped five at a time, by fixing one of the two concentrations. In Figure 3.22, the HAMA concentrations are fixed, and the PEGDMA concentrations are compared for each value of HAMA. In Figure 3.23, the PEGDMA concentrations are fixed and HAMA concentrations are compared. The graphs are presented as a fixed maximum value on the y axis, to better compare the graphs to each other, and the second is with an adjusted y scale, to be better visualise the individual curves in the plots.

The graphs in Figure 3.22 were plotted with a fixed HAMA concentration and a varying PEGDMA concentration. For all fixed concentrations of HAMA, the degradation decreased with increasing PEGDMA concentration, similar to the pilot study. This was due to the increase in the crosslinking density of the gels, which slowed down the effect of the enzyme.

The graphs in Figure 3.23 were plotted with a fixed PEGDMA concentration and a varying HAMA concentration. For all fixed concentrations of PEGDMA, the degradation decreased with increasing HAMA concentration, similar to the pilot study. This was due

to the increase in the crosslinking density of the gels, which slowed down the effect of the enzyme.

Therefore, the degradation behaviour was as predicted from the pilot studies and demonstrated that the degradation rate and the total amount of degradation could be varied depending on the concentration of both HAMA and PEGDMA.

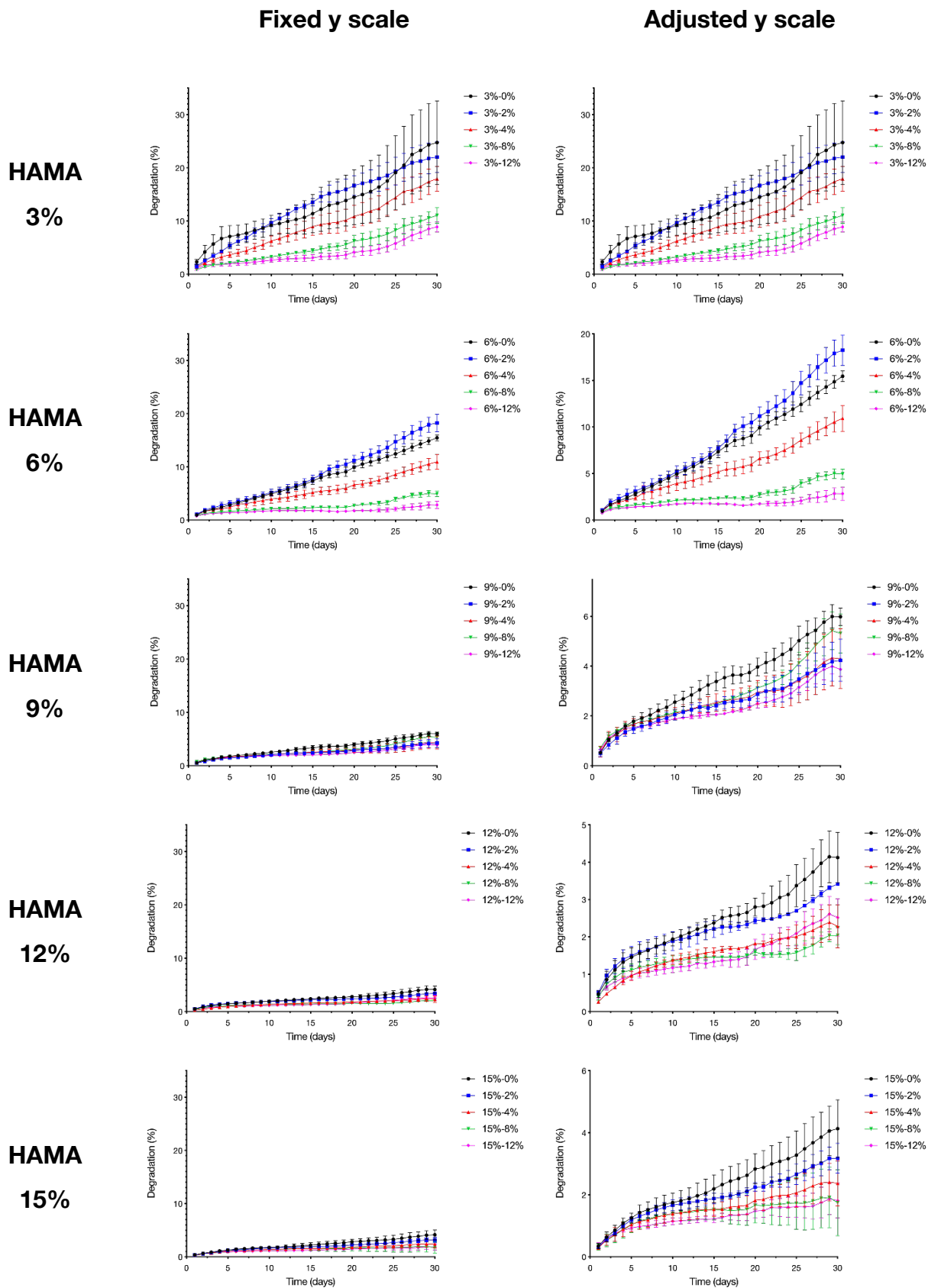


Figure 3.22: Cumulative degradation percentage of the fixed HAMA concentrations with varying PEGDMA concentrations. Values were plotted over the course of the 30-day experiment, plotted with a fixed and adjusted y scale (N=3, n=3).

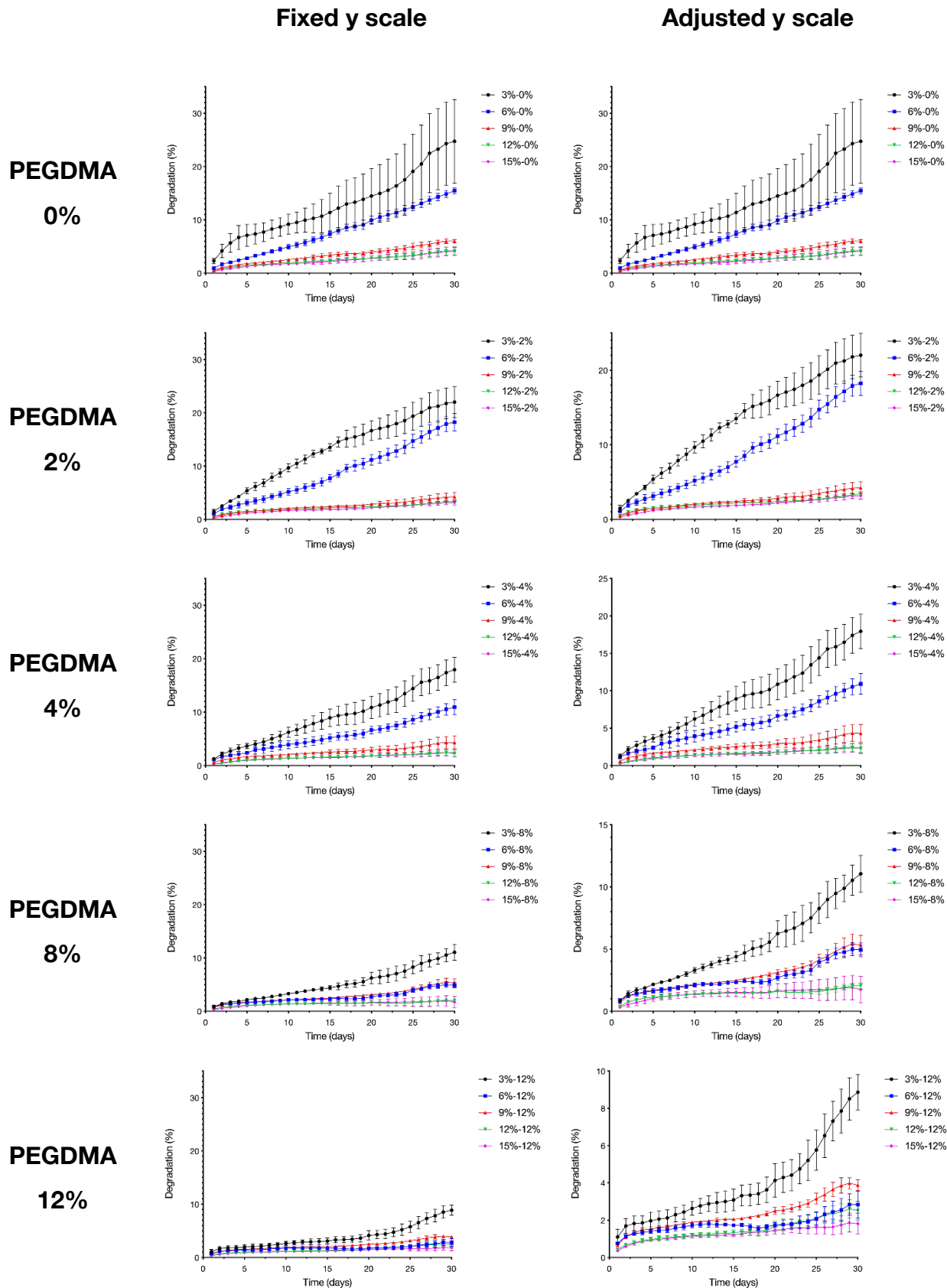


Figure 3.23: Cumulative degradation percentage of the fixed PEGDMA concentrations with varying HAMA concentrations. Values were plotted over the course of the 30-day experiment, plotted with a fixed and adjusted y scale (N=3, n=3).

Even though the trends of the graphs plotted in Figures 3.22 and 3.23 did match the pilot study and the expected degradation behaviour as described in the literature, there was a discrepancy in the full study compared to the pilot study. Looking more closely at the values of degradation percentage achieved in the full study, they are all lower than expected when considering the results of the pilot study. To directly compare them to the pilot study, the 4 common concentrations tested were plotted for the initial 5 days of degradations and can be seen in Figure 3.24. For all 4 concentrations, the pilot degradation values are at least twice as high as the ones from the full study after 5 days.

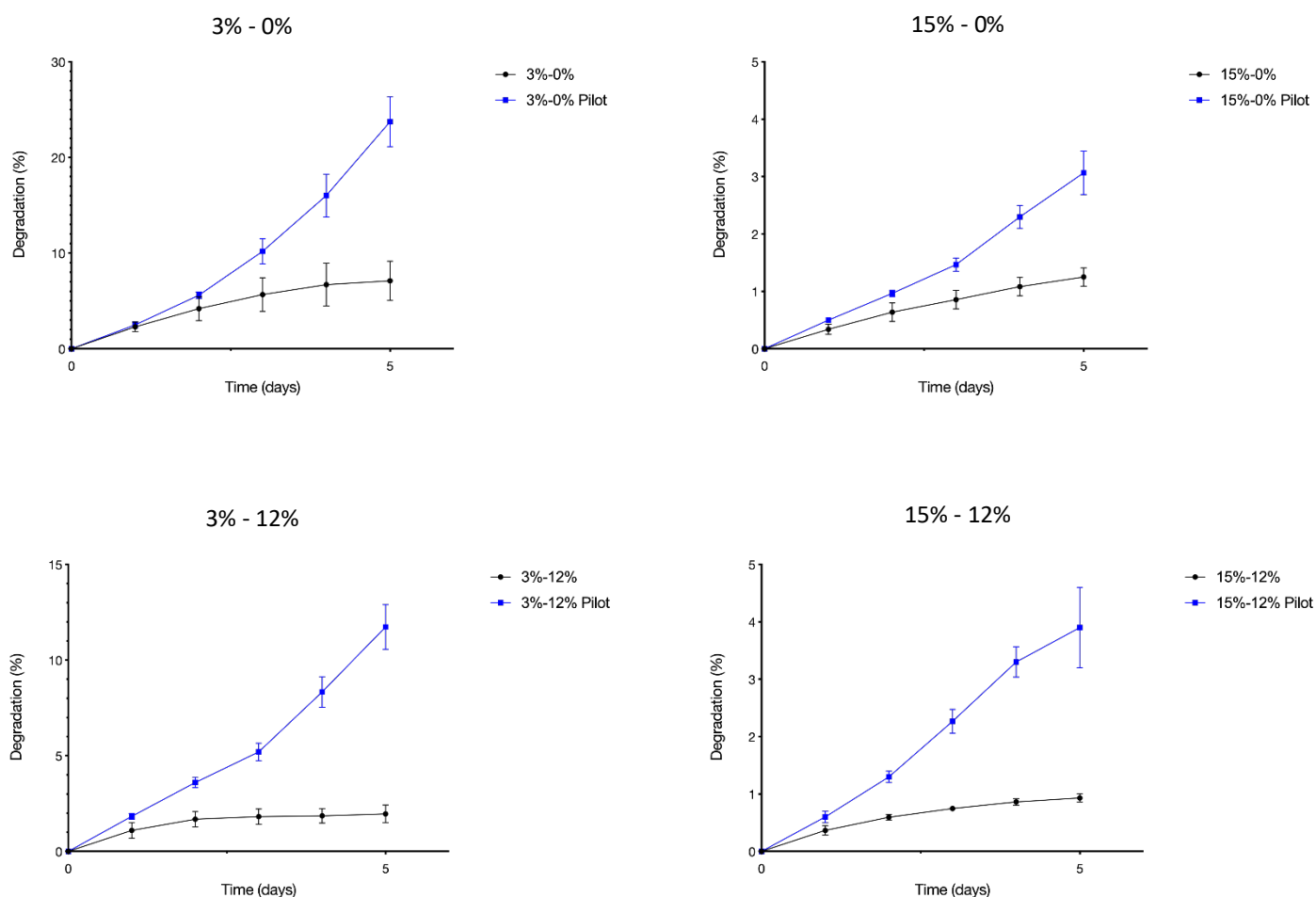


Figure 3.24: Cumulative degradation percentage of the 4 concentration conditions. The plotted data was collected over the course of 5 days for both the pilot and the full experiment (N=3, n=3).

Furthermore, when looking at the gels by the end of the 30-day experiment, it was clear that the 3% HAMA – 0% PEGDMA gels were fully degraded. However, looking at the values in the graph, the hydrogels with this concentration only degraded up to 25% after 30 days, which does not align with the observations. In the pilot experiment, the hydrogels with the matching concentrations had already degraded by 25% after only 5 days.

It is unclear why the calculated degradation values of the full study appear to be lower than what is observed in the pilot study. The only difference between the 2 experiments was that the hyaluronidase control, used to subtract any background signal, was added only to the 384-well plate and was a fresh enzyme solution. In contrast, in the full study, the control was placed in the 48-well plate and was incubated similarly to the hydrogel solutions and was collected daily.

The reason for the modification was to ensure that the control was treated in the same way that the hydrogels were. However, the absorbance values for the hyaluronidase controls values were lower for the pilot study. The increased values in the full study meant that a larger background signal was subtracted from the measured values, and that the calculated mass was lower, thus decreasing the values of degradation calculated.

The reason the measured absorbances for the hyaluronidase control were higher for the full study was unclear. The enzyme used was from the same batch, and at the same concentration, the buffer used was also the same, as well as the Micro BCA assay kit and the plate reader. The only variable was the incubation of the solution for 24 hours with the rest of the plate.

3.5 Conclusion

The HAMA – PEGDMA – LAP hydrogels were characterized to determine the optimal polymer concentrations for the desired physical properties of the scaffold, with four different studies: swelling, rheology, diffusion, and degradation.

The results of the swelling experiment demonstrated that the percentage of water uptake was tuneable by varying the concentrations of the gel components, but that this effect plateaued after a certain concentration. The results from the PEGDMA and HAMA screening experiments were expected, as increasing the concentrations of HAMA and PEGDMA causes an increase in crosslinking density, which in turn causes a decrease in hydrogel porosity, as previously reported in the literature [124], [141]. This indicates that the hydrogel was not able to expand to the same extent with higher concentrations of materials and could not uptake as much water as the gels with lower concentrations when submerged in a fluid [190], [209]. However, there was less evidence of this decreasing water uptake with higher concentration of materials, and the tunability of swelling and water uptake could only be controlled to a certain degree. The rate of water uptake was not studied in this experiment as this parameter was less relevant for the overall process of making patterned hydrogel scaffolds. Furthermore, having a range of PEGDMA and HAMA concentrations that result in minimal gel swelling is a positive result, because it suggests the possibility of having hydrogels with a range of degradation rates, all of which are minimally swelling.

The results of the rheology experiments demonstrated that a range of tuneable hydrogels can be fabricated, with storage moduli that can be controlled by varying the concentrations of either HAMA or PEGDMA. It was observed that with increasing polymer concentration, the storage modulus increases, due to the increasing crosslinking density of the hydrogel [124]. The LAP screening experiment indicated that 1% LAP produced the hydrogels with the highest storage modulus for fixed HAMA and

PEGDMA concentrations, and this concentration was used for the rest of the experiments.

The diffusion experiment studied was a pilot experiment that only included 5 of the possible hydrogel formulations, and only 2 dextran molecular weights. However, the results were still informative and gave an indication on the diffusion behaviour of molecules within hydrogels of different concentrations. Further studies with the other hydrogel formulations and a wider range of dextran molecules should be performed to determine the limit of diffusion.

The degradation experiments demonstrated that it was possible to tune the degradation rate of the hydrogels by varying the concentrations of HAMA and PEGDMA, whereby lower concentrations of polymer, degradation rates were increased, and the inverse was true as well. Additionally, the enzyme concentration used was equivalent to the measured concentration in vitreous extracted from human eyes [208]. However, enzyme concentration may vary *in vivo* for both animal and human models.

These experiments indicated that the 9% HAMA – 4% PEGDMA – 1% LAP hydrogel formulation was the best one to use during mould fabrication optimisation that will be discussed in Chapter 4. All these studies were conducted with a bulk hydrogel instead of the patterned hydrogel and should be repeated in the future on the final patterned scaffold to determine how the pattern would affect all the results obtained.

Chapter 4: Scaffold fabrication: optimising the channel geometry and the scaffold demoulding process

With the findings of Chapter 3 determining an optimal hydrogel formulation, further optimisation was required to successfully fabricate the scaffold with the desired pattern, particularly with the specific channel geometry that was designed, in a repeatable and reproducible way.

This chapter is a continuation of Chapter 2 and provides further scaffold fabrication optimisation. It will focus on producing the desired channel geometry and aims to make the scaffold demoulding process reproducible and semi-automated. The results of this chapter were obtained in collaboration with Dr. Eoghan Cunnane.

4.1 Optimizing filling:

4.1.1: Testing the surface treatment:

Chapter 2 ended with a confocal image of a demoulded patterned hydrogel that highlighted a challenge in filling the negative print with the hydrogel. Another example of an unsuccessful filling hydrogel was dried and imaged on the SEM to visualise the hydrogel from another angle. Looking at Figure 4.1, it appears that the hydrogel precursor solution did not infiltrate the negative mould past the bottom of the stems.

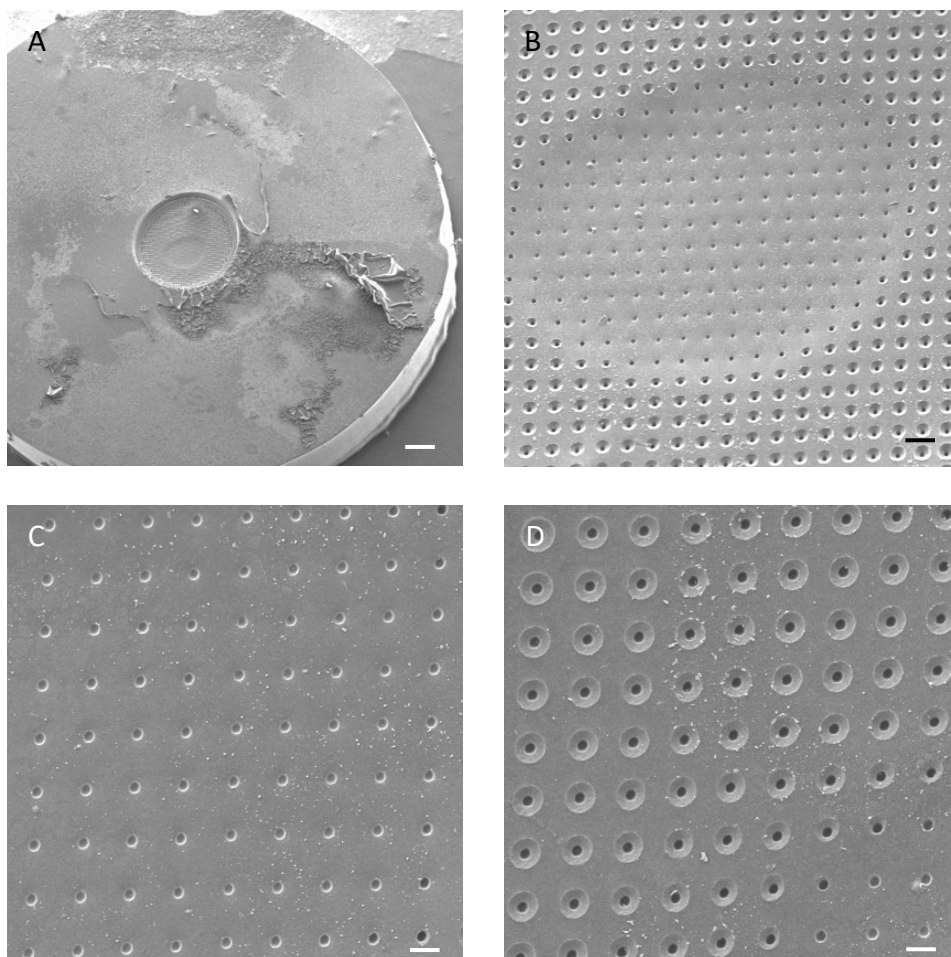


Figure 4.1: SEM images of a demoulded hydrogel. Demoulding was carried out by Dr. Eoghan Cunnane. Images were taken at 25X (A), 250X (B), and 500X (C) and (D) magnifications, using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bars: (A): 200 μm , (B): 20 μm , (C) and (D): 10 μm .

In addition to imaging the hydrogel, the negative print it was demoulded from was also imaged on the SEM. Looking at the images in Figure 4.2, it was clear that the columns and stems were intact, and the entire hydrogel came off, meaning that the moulds could be re-used.

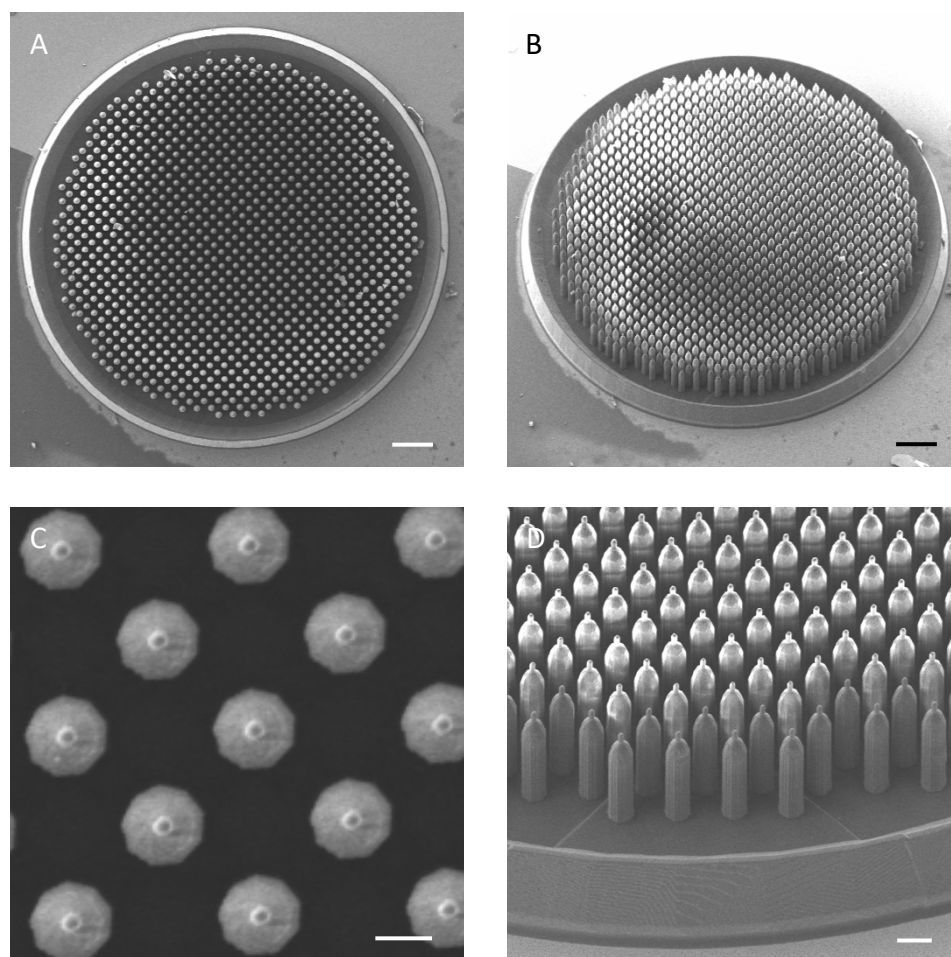


Figure 4.2: SEM images of a negative print following demoulding. Images were taken at 60X (A) and (B), 1000X (C), and 250X (D) magnifications, using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bars: (A) and (B): 100 μm , (C): 10 μm , and (D): 20 μm .

As the negative print was silane treated to make the surface more hydrophobic, it is possible that it was interfering with the filling step. To see if the silane treatment step was necessary or could be removed from the process, demoulding was attempted with an un-silane treated negative print. The print was still plasma treated, which meant that the surface was hydrophilic.

The gel precursor solution with concentrations 9% HAMA (with Cy5 labelled HAMA added at a ratio of 1:100), 4% PEG and 1% LAP was prepared as described in section 3.1.2. Lyophilized HAMA and PEGDMA were dissolved in 1X PBS containing 1% LAP to prepare the precursor solution. The LAP solution was first added to the PEG and vortexed until the PEG was fully dissolved. Following that, the new solution mixture was pipetted on top of the HAMA and left overnight at 4°C to dissolve the lyophilised HAMA.

Similarly, to the protocol described in section 2.4.6., 20 µL of the prepared gel precursor solution was added into the reservoir created around the negative print. The substrate was then placed on the spin coater for 10 minutes at 1000 rpm to spread the excess precursor solution away from the print, and only leave a layer with the thickness of the print. Once completed, a pre-treated cover slip, was added on top of the gel and the setup was placed under the UV lamp for 10 minutes to cure the gel. Once fully polymerised, the hydrogel, still sandwiched between the mould and the cover slip, was left to air dry for 30 minutes.

Once the drying step was completed, the cover slip was removed, and a part of the hydrogel did come off with the cover slip. However, the hydrogel tore around the print, and the remaining section of the hydrogel stuck to the negative print. The print with the gel still attached to it was imaged on the SEM to better visualise the hydrogel and how the printed micro-structures were affected. Looking at Figure 4.3, it seemed that the gel did infiltrate the print more deeply when it was not silane treated. It did appear that the columns were deformed slightly, and were pulled in towards each other, probably due to the hydrogel drying. The stems did seem intact. This experiment was repeated 4

several times and yielded the same results. Therefore, it can be concluded that plasma treatments on its own is not an option for demoulding the hydrogels, and silane treatments are needed to be able to demould the hydrogel successfully.

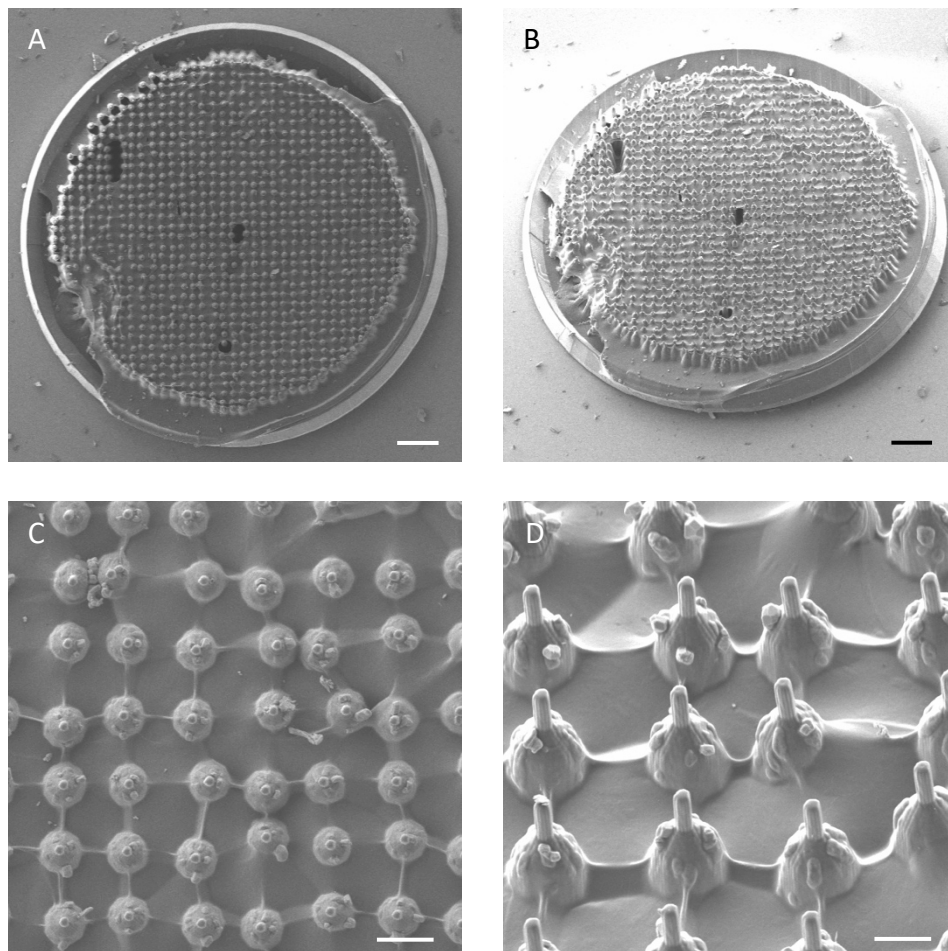


Figure 4.3: SEM images of an unsuccessful demoulding. Images were taken at 60X (A) and (B), 500X (C), and 1000X (D) magnifications, using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bars: (A) and (B): 100 μm , (C): 20 μm , and (D): 10 μm .

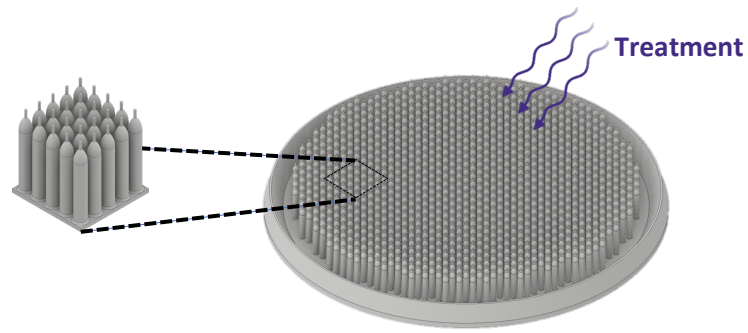
4.1.2: Optimizing the hydrogel filling process:

As silane treatments are necessary to demould the hydrogel and be left with an intact negative print, the hydrogel filling process needed to be adjusted to make sure the hydrogel infiltrates the negative mould fully. Previously, spin coating was used for the purpose of getting flat thin gels, however it seemed like using spin coating as a technique only caused the hydrogel precursor solution to be expelled radially, instead of down into the print. Therefore, the spin coating step was removed and replaced with adding a 60 g weight on top of the cover slide, as illustrated in Figure 4.4. Specifically, the negative print on the substrate was plasma treated and silane treated. Then, 20 μL of the prepared gel precursor solution was added into the reservoir created around the negative print with the plastic sheet and a 6 mm punched hole. Then, the pre-treated cover slip was added directly on top of the gel and a clear glass 60 g weight was added on top of that to force the precursor solution into the mould. The setup was then placed under the UV lamp for 10 minutes to cure the gel. Once fully polymerised, the hydrogel, still sandwiched between the mould and the cover slip, was left to air dry for 30 minutes.

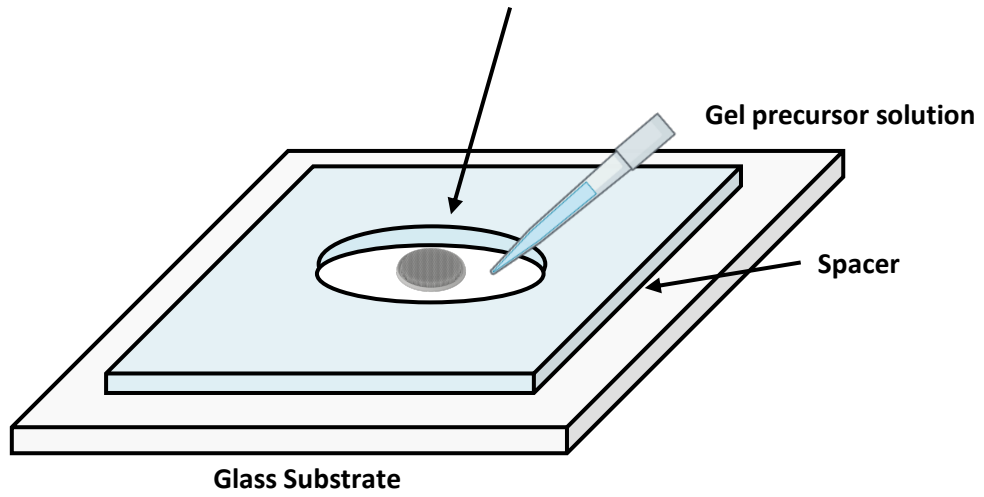
Once the drying step was completed, the cover slip was carefully removed, and the hydrogel successfully came off with the cover slip. This confirmed again that the silane treatment was required to successfully demould the hydrogel scaffold from the negative print.

To see if the modified hydrogel filling process was successful in driving the hydrogel precursor solution to the bottom of the negative print, the demoulded patterned hydrogel was rehydrated and imaged on a confocal microscope and can be seen in Figure 4.5.

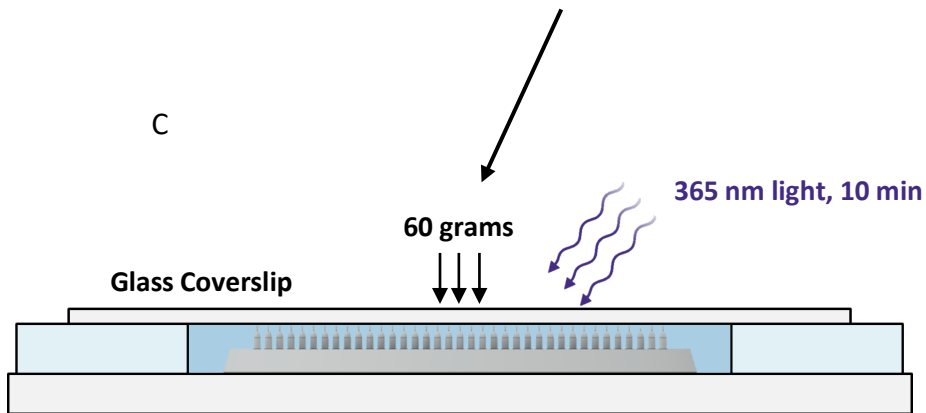
A



B



C



D

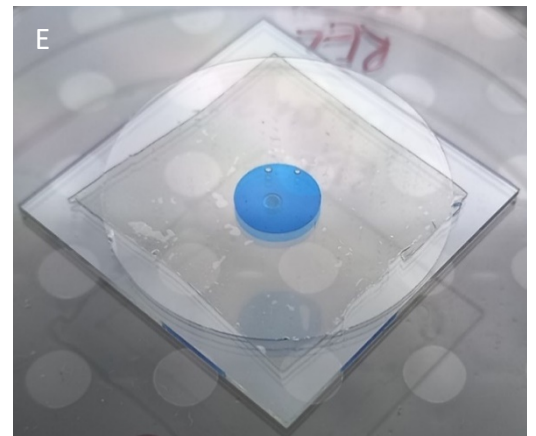
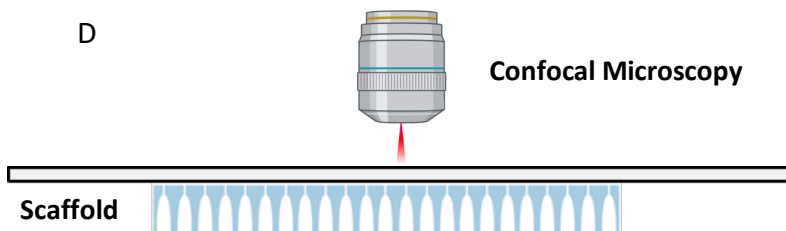


Figure 4.4: Modified hydrogel filling process. (A): The negative printed mould is plasma and silane treated. (B): The gel precursor solution is added into the reservoir. (C): The glass cover slide and the 60 g weight are added, and then the setup is UV crosslinked for 10 minutes. (D): The demoulded cover slip with the attached patterned hydrogel was imaged on the confocal microscope. (E): Picture of the setup. The figure can be seen in the page above.

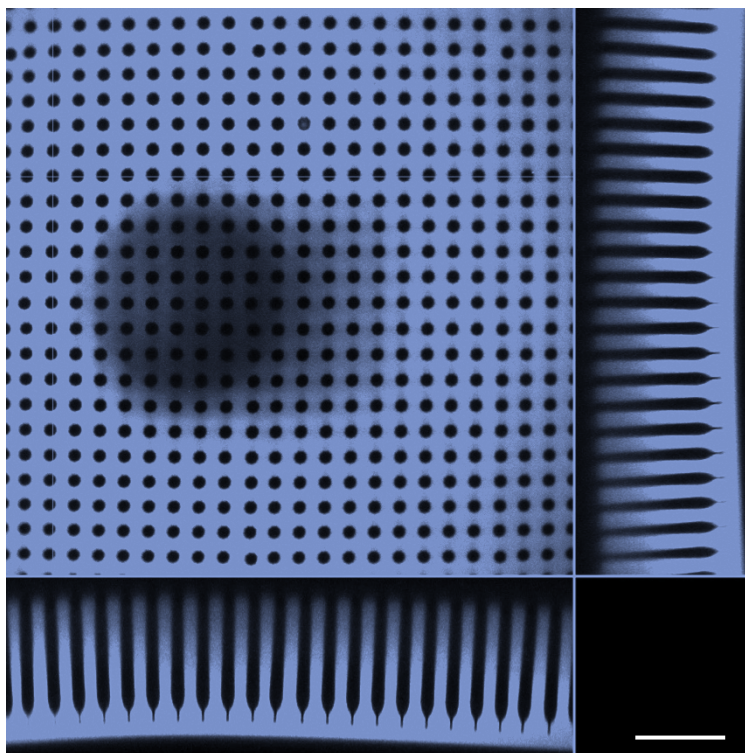


Figure 4.5: Confocal z stack image of a demoulded micro-patterned hydrogel. The hydrogel successfully contained the patterned channels. The demoulding and microscopy were performed by Dr. Eoghan Cunnane. Scale bar 100 μm .

Looking at the confocal image in Figure 4.5, it could be seen that the hydrogel successfully contained the full patterned channels, meaning that the modifications made to the filling process were effective. The hydrogel contained identical repeating channels including the thin stems.

With the success of the filling method came another challenge. Looking more closely at the image in figure 4.5, it could be seen that the holes at the bottom of the scaffold were not through holes. Instead, there was a layer of extra gel material. In the next section, this challenge will be addressed in more details.

The final thing to assess was the state of the patterned scaffold after a longer period of incubation to check the stability of the pattern and to verify that minimal swelling occurred. The patterned scaffold was rehydrated and imaged on the confocal microscope within one hour of its demoulding (Figure 4.6 (A)). It was then left incubating for 1 week in PBS and imaged again (Figure 4.6 (B)). From the figure, it can be seen that the patterns were preserved and there was no evidence of swelling or change in the channel dimensions, validating the choice of material as well as the concentration chosen for these optimisation steps.

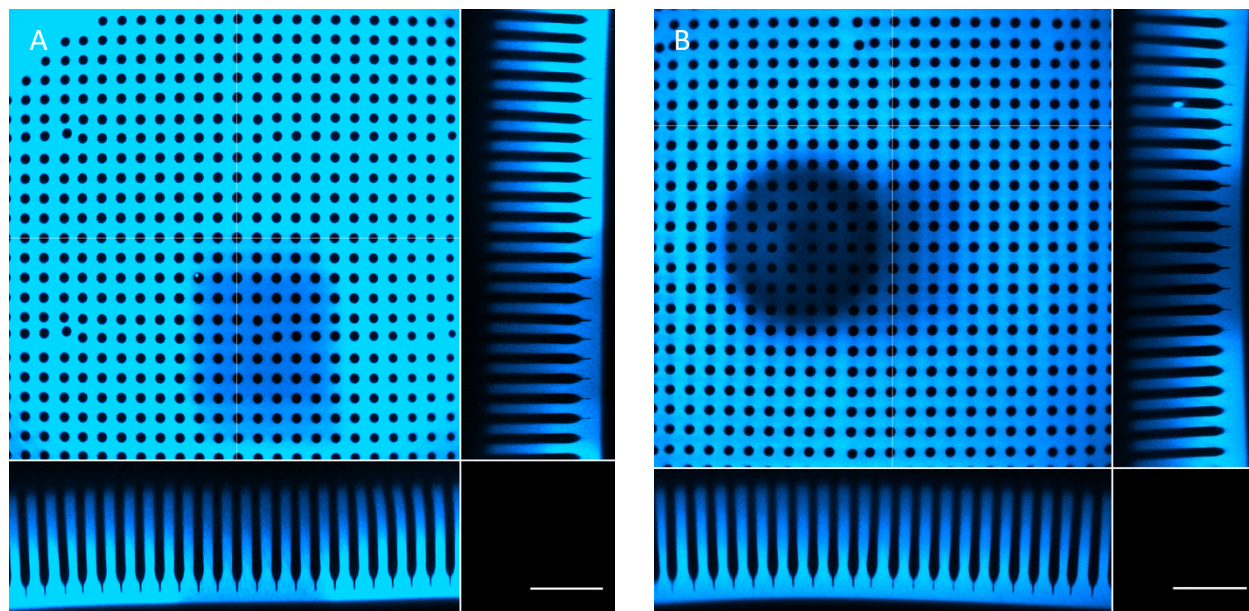


Figure 4.6: Confocal z stack image of a demoulded micro-patterned hydrogel at two time points. The hydrogel was imaged after 1 hour (A) and after 1 week (B). The demoulding and microscopy were performed by Dr. Eoghan Cunnane. Scale bar 100 μm .

4.2 Optimizing the through holes:

4.2.1 Changing to a silicone spacer:

In previous experiments, a plastic sheet was used as a spacer to create a reservoir for the hydrogel precursor solution. Despite showing some success, it was suboptimal as it did not stick to the glass substate and cover slip, and therefore did not create a perfect seal. Instead, it left visible air gaps as depicted in Figure 4.7. It was hypothesised that these gaps were affecting the total height of the spacer and subsequently the total thickness of the patterned hydrogel, thereby resulting in the excess material observed above the stems of the channels.

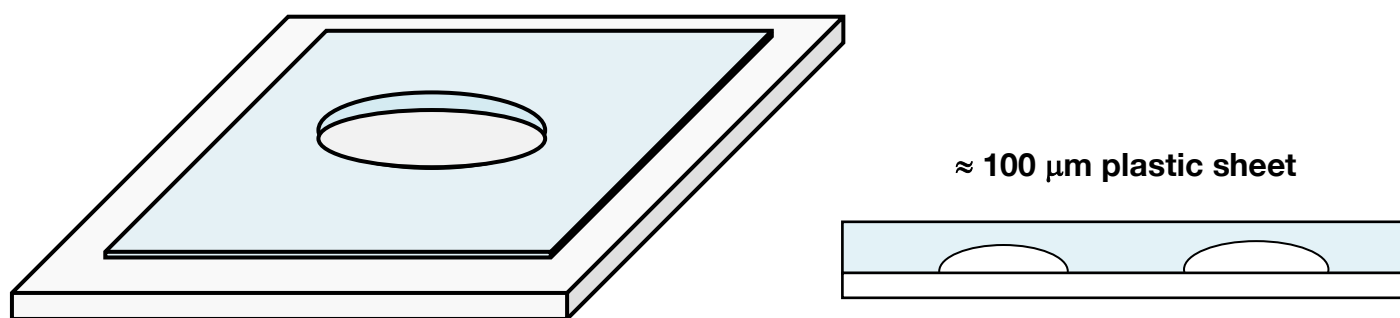


Figure 4.7: Schematic of the 100 μm plastic sheet spacer. Schematic of the drawbacks of the plastic sheet spacer and the possible air gaps formed between the spacer and the glass substrate.

To overcome this challenge, the plastic spacer was replaced with a silicon spacer (Purchased from Silex Silicones Ltd). As these spacers came in very limited dimensions, there was no spacer with the exact height of the negative print (105 μm). Instead, the closest option to achieve the full height of the negative print was to use a 100 μm spacer, in addition to a 50 μm spacer, and adjust the height of the negative print to 150 μm to match the spacers. Therefore, the columnar structures were increased to 120 μm in height, and the base of the print was increased to 30 μm (Figure 4.8).

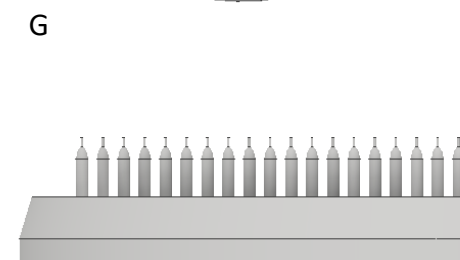
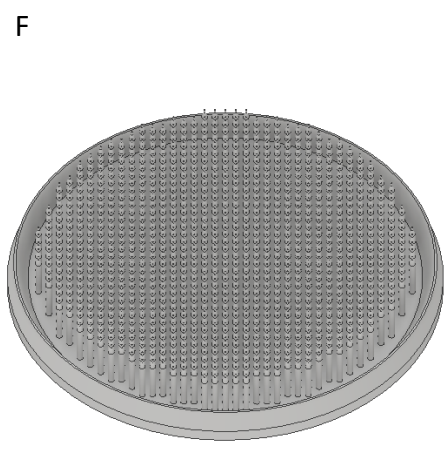
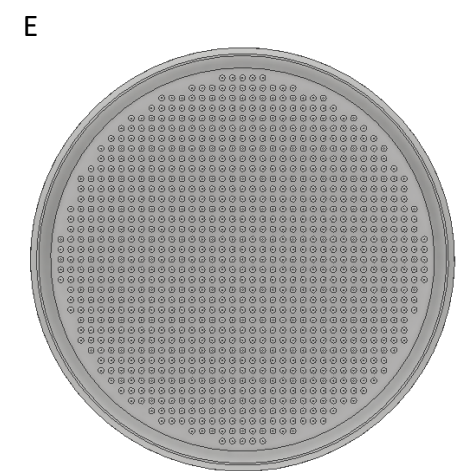
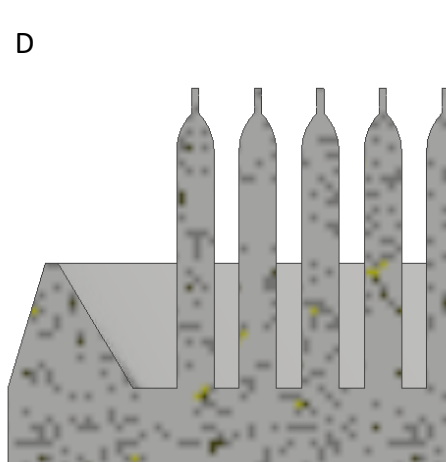
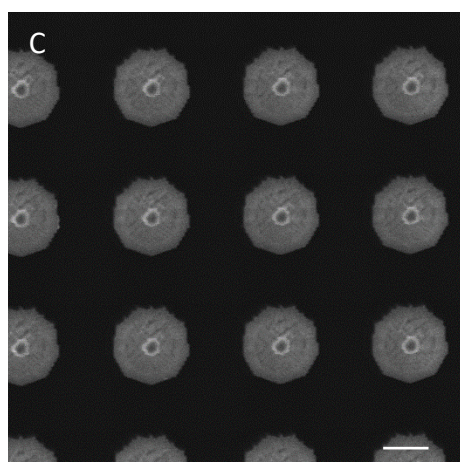
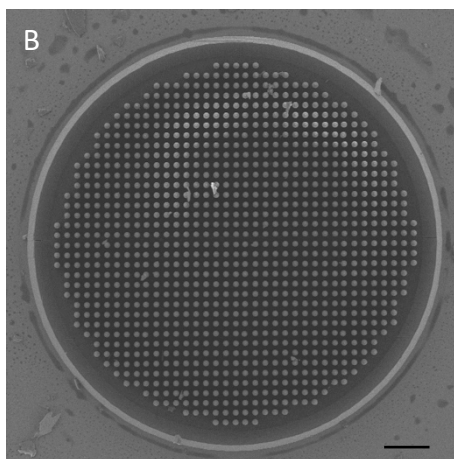
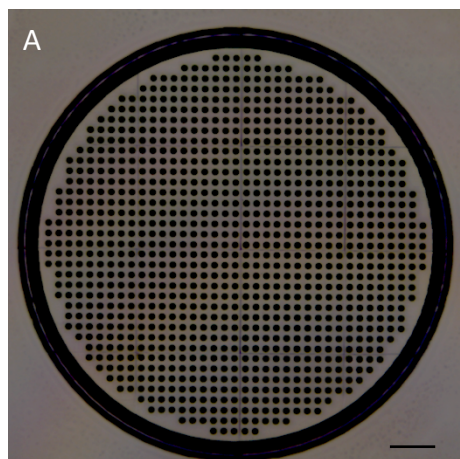


Figure 4.8: Circle area print with rectangular array and 150 μm height.

(A): Light microscope image taken after the cleaning step, using an EVOS XL core microscope (Thermofisher).

(B): SEM image taken at 150X magnification.

(C): SEM image taken at 2000X magnification.

(D): Cross section view of the 3D model.

(E): Top view of 3D model.

(F): Tilted view of 3D model.

(G): Side view of 3D model.

All SEM images were taken using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV.

Scale bars:

(A) and (B): 150 μm

(C): 10 μm

The 100 μm and 50 μm silicon spacers were carefully assembled one on top of the other. A 6 mm hole was punched out of the two overlapping sheets before they were placed around the already plasma and silane treated negative print, to create a reservoir. Then, 20 μL of the prepared gel precursor solution (9 % HAMA with Cy5 labelled HAMA added at a ratio of 1:100, 4% PEG and 1% LAP) was added into the reservoir. Following that, the cover slip was added directly on top of the gel and a clear glass 60 g weight was added on top of that to force the precursor solution into the mould. The setup was then placed under the UV lamp for 10 minutes to cure the gel.

Once fully polymerised, the cover slip was carefully removed, and the hydrogel successfully came off with the cover slip. To see if the modified spacer allowed for the formation of through holes, the demoulded patterned hydrogel was placed in PBS and imaged on a confocal microscope (Figure 4.9). Unfortunately, the issue persisted and there was still excess material on top of the stems.

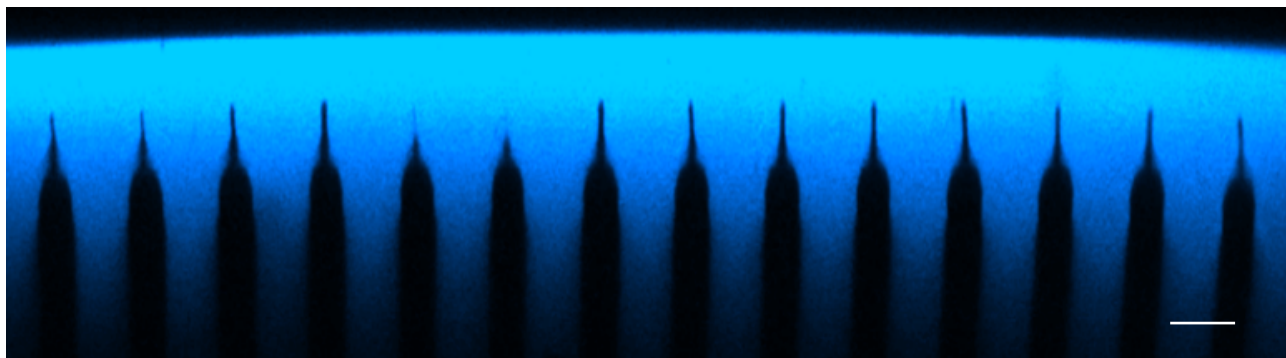


Figure 4.9: Confocal z stack image of a demoulded micro-patterned hydrogel. The demoulding and microscopy were performed by Dr. Eoghan Cunnane. Scale bar 20 μm .

Despite the individual silicone sheets having an accurate thickness, placing them on top of each other has the potential to cause thickness inconsistencies due to trapped air or sheet puckering, as depicted in Figure 4.10. These inconsistencies were again enough to affect the total height of the spacer and subsequently the total thickness of the patterned hydrogel.

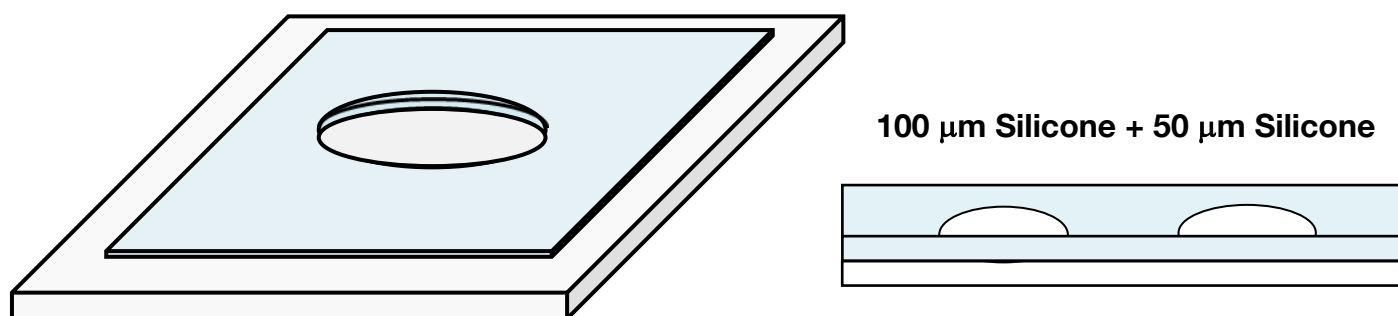


Figure 4.10: Schematic of the two spacers placed on top of each other. Schematic of the 100 and 50 μm spacers placed on top of each other, and the possible air gaps formed between them.

In addition to imaging the demoulded hydrogels, the printed negative moulds were also imaged on the light microscope and SEM microscope to validate the success of the process as well as check if there was any damage to the prints. The images of both a rectangular and hexagonal array of these prints can be seen in Figure 4.11. The images confirm that the print is still intact after the demoulding and there is no visible damage to the columns or the stems. This is important because it means that the print can be re-used for future experiments.

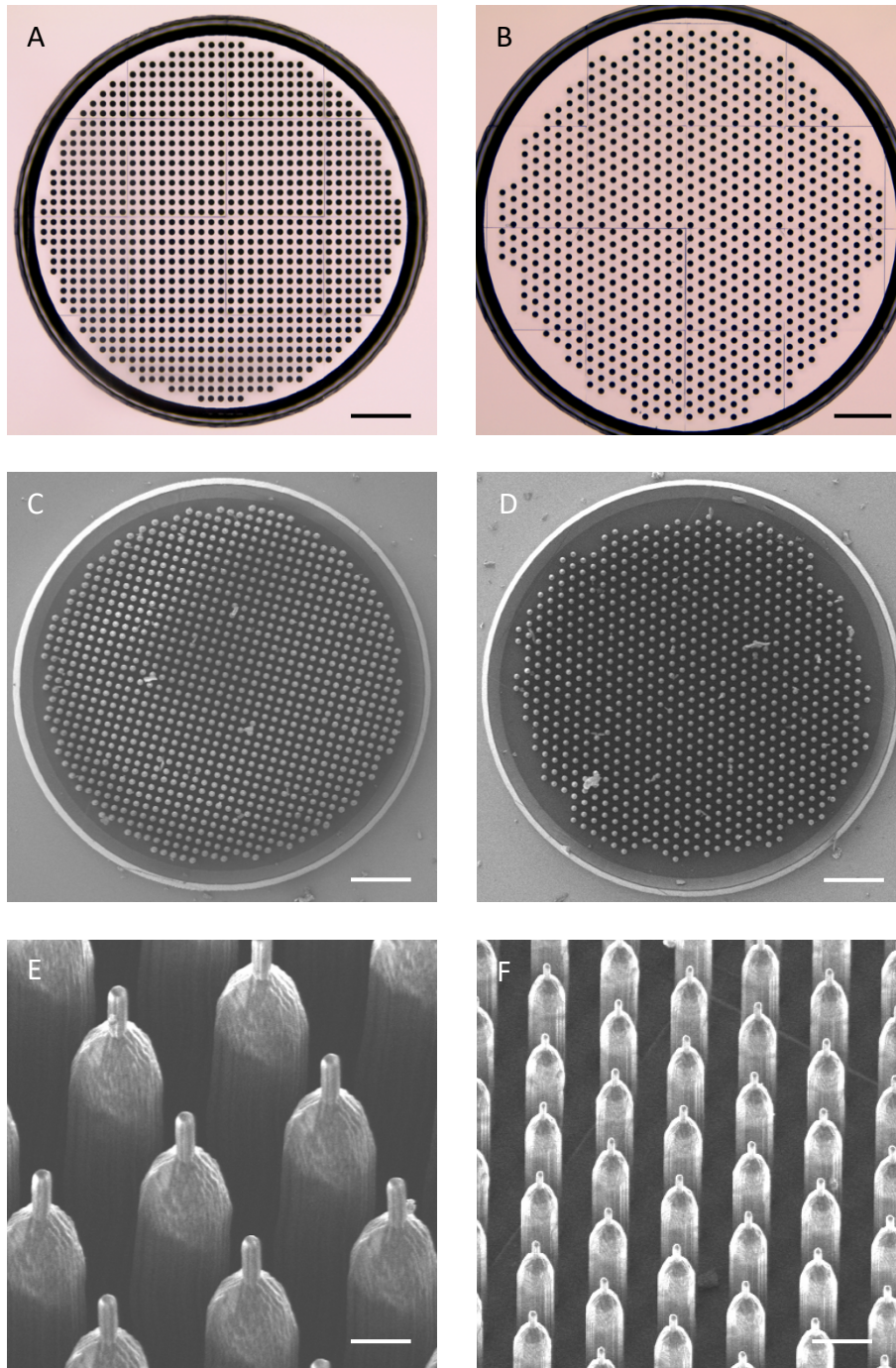


Figure 4.11: Images of a negative print following demoulding. (A) and (B): Light microscope images taken of successfully demoulded negative print, using an EVOS XL core microscope (Thermofisher). (C-F): SEM images of a negative print following demoulding, taken at 60X (C) and (D), 1000X (E), and 250X (F) magnifications, using the Zeiss Auriga Cross Beam. (A), (C) and (E) are images of a rectangular array, (B), (D) and (F) are images of a hexagonal array. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bars: (A), (B), (C) and (D): 150 μm , (E): 10 μm , and (F): 20 μm .

4.2.2 Increasing the print and spacer heights:

To ensure the accuracy of the spacer height, a 200 μm silicone spacer was purchased from the same company as it was the next size up from 100 μm available (Figure 4.12). Accordingly, the negative print needed to be modified to a total height of 200 μm to match the spacer. The columns were kept at 120 μm high, and the base increased to 80 μm thick.

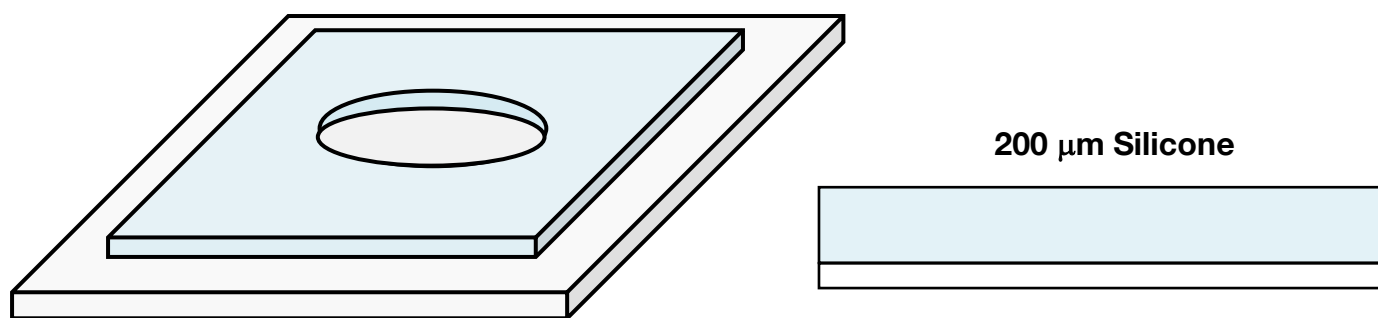


Figure 4.12: Schematic of the 200 μm spacer. Schematic of the 200 μm spacer placed on top of the glass substrate with no air gaps formed between them.

Similarly, the 200 μm silicon spacer, with a 6 mm hole punched out, was placed around the already plasma and silane treated negative print, to create a reservoir. Then, 20 μL of the same prepared gel precursor solution (9% HAMA with Cy5 labelled HAMA added at a ratio of 1:100, 4% PEG and 1% LAP) was added into the reservoir. Following that, the cover slip was added directly on top of the gel and a clear glass 60 g weight was added on top of that to force the precursor solution into the mould. The setup was then placed under the UV lamp for 10 minutes to cure the gel. Once fully polymerised, the hydrogel, still sandwiched between the mould and the cover slip, was left to air dry for 30 minutes.

Once the drying step was completed, the cover slip was carefully removed, and the hydrogel successfully came off with the cover slip. To see if the modified spacer allowed for the formation of through holes, the demoulded patterned hydrogel was placed in PBS and imaged on a confocal microscope and can be seen in Figure 4.13. Unfortunately, the issue was still present, however the amount of excess material on top of the stems was less than what was observed previously.

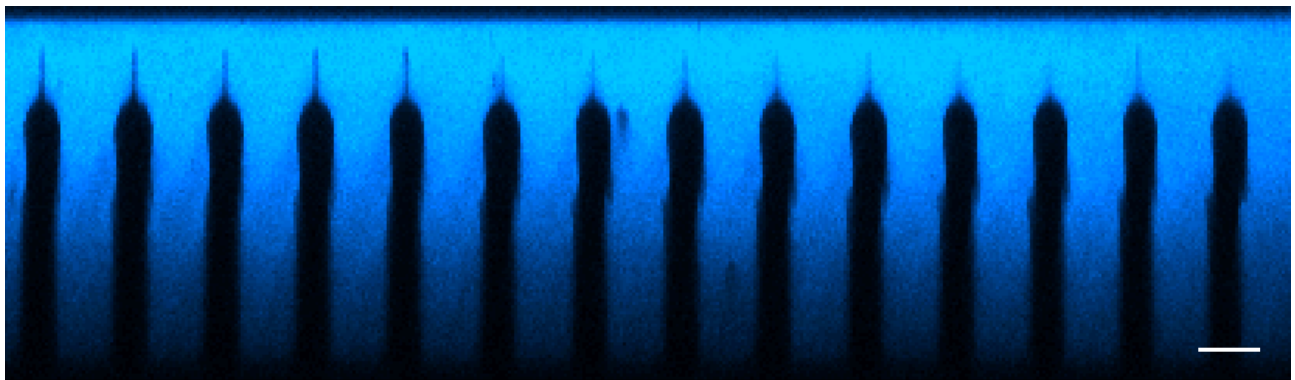


Figure 4.13: Confocal z stack image of a demoulded micro-patterned hydrogel. The demoulding and microscopy were performed by Dr. Eoghan Cunnane. Scale bar 20 μm .

The confocal image did expose an unexpected new challenge. Looking at the image in Figure 4.14, it appeared that the stems were not perfectly straight, and there was some irregularity in the pattern. To understand the cause of this issue, the negative print was imaged on the SEM (Figure 4.14).

Looking at the SEM image, it was clear that the abnormal column shapes in the patterned hydrogel was due to the irregularities in the negative print, and they were transferred to the hydrogel when it was moulded. The columns appeared to be printed in two layers, and they did not align properly.

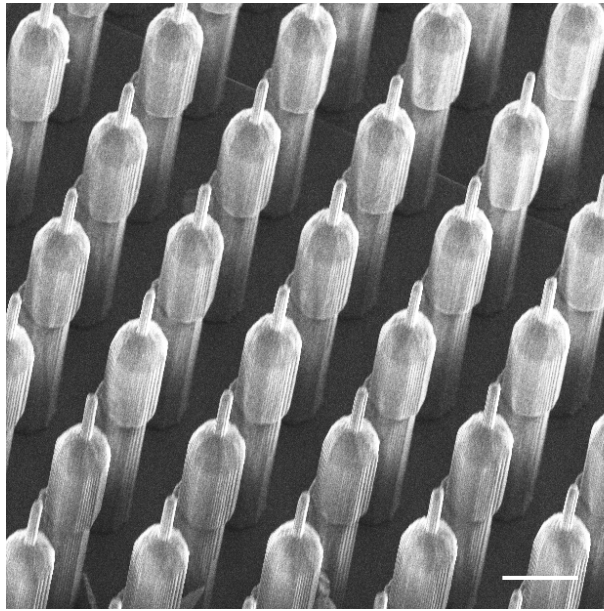


Figure 4.14: SEM images of a 200 μm negative print. SEM image was taken at 1000X magnification, using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bar 20 μm .

To understand where this originated from, it is useful to look back at Chapter 2, section 2.4.2, where the Nanoscribe printer is described. As mentioned there, as the print is larger than the print field diameter of the 3D medium feature solution set chosen (400 μm in diameter), the Describe software divides the 3D model into smaller blocks. When the print height was less than 150 μm , the z directional dimension for each block was not relevant because the software's default value for that dimension was 150 μm . Therefore, when the print height was increased to 200 μm , the Describe software divided the print into two layers of blocks, the first with a height of 150 μm and the second with the remaining 50 μm .

Although the Nanoscribe printer claims to successfully print models of this dimensions, and even larger, without any issues in aligning and stitching the blocks together, it was

not the case for this specific 3D model. The SEM images showed that there was no issue in stitching blocks in the x and y directions, only in the z direction.

After consulting the Nanoscribe team and reading more into how the printer works, it became clear that the issue originated from how the printer finds the interface on the glass substrate. It turned out that when it prints one layer of blocks, it finds the interface each time it begins printing that block, making the location of the print very accurate. However, when it prints more than one layer of blocks, it only finds the interface once at the beginning of the whole print and uses that value as an assumption for the interface values at all the different locations along the surface of the substrate. This slight difference was all it took to affect the quality of the print and the alignment of the two layers of blocks. The solution was simple: to increase the block dimension in the z direction to 250 μm . With that small adjustment, the 3D model was printed successfully, as seen in Figure 4.15.

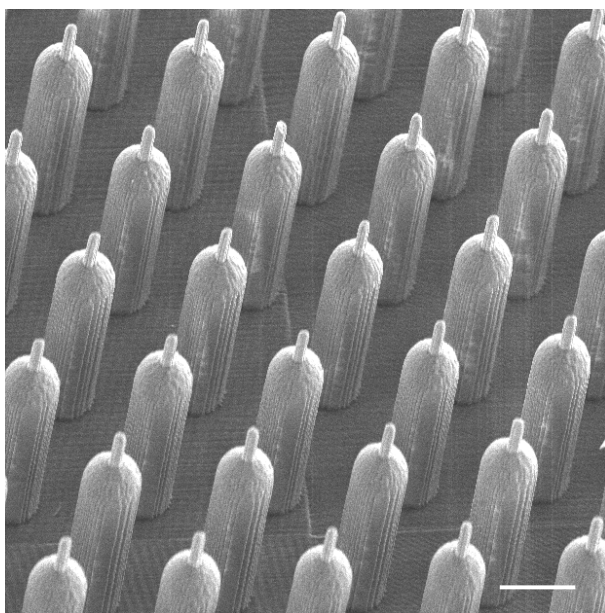


Figure 4.15: SEM images of a 200 μm negative print. Images were taken at 1000X magnification, using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bar 20 μm .

4.2.3 Optimizing the height of the print:

After solving the printing challenge, the next step was to attempt to demould the hydrogel again. Since using a negative print with a total height of 200 μm still resulted in excess material on top of the stems, the height of the print was further increased. Looking back at Figure 4.13, the average thickness of the extra layer of material on top of stems was measured and came to approximately 10 μm . Therefore, as a first attempt, the total height of the print was increased to 210 μm by increasing the thickness of the base to 90 μm and keeping the columns at 120 μm . Once printed, the negative mould was plasma and silane treated to ready the surface for the demoulding step. Using the same 200 μm silicone spacer, and the same gel precursor solution (9% HAMA with Cy5 labelled HAMA added at a ratio of 1:100, 4% PEG and 1% LAP), the hydrogel scaffold was fabricated and demoulded. The patterned scaffold was imaged on the confocal microscope and can be seen in Figure 4.16.

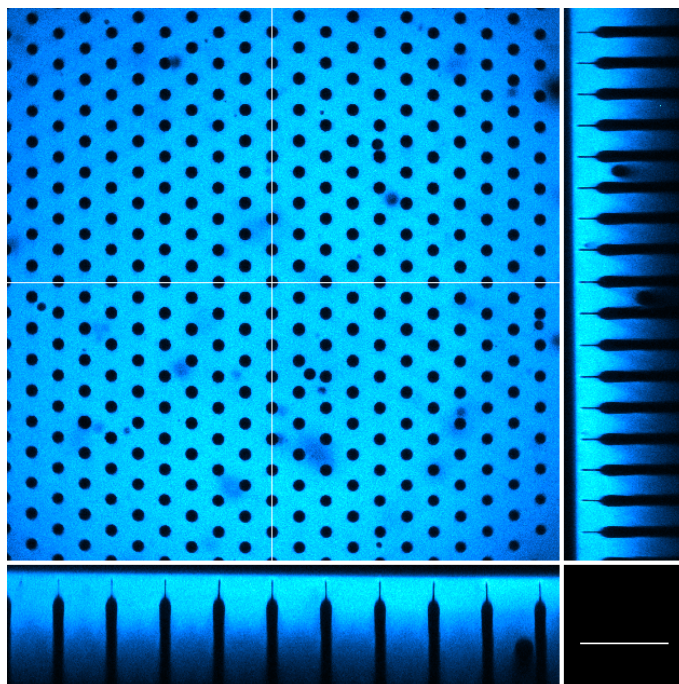


Figure 4.16: Confocal z stack image of a demoulded micro-patterned hydrogel. The demoulding and microscopy were performed by Dr. Eoghan Cunnane. Scale bar 100 μm .

There was a visible improvement in the scaffold and the excess material on top of the stems was reduced, however a small amount of excess material remained.

The next attempt consisted of increasing the total height of the print to 215 μm by increasing the thickness of the base to 95 μm and keeping the columns at 120 μm . Once printed, the negative mould was plasma and silane treated to ready the surface for the demoulding step. Using the same 200 μm silicone spacer, and the same gel precursor solution (9% HAMA with Cy5 labelled HAMA added at a ratio of 1:100, 4% PEG and 1% LAP), the hydrogel scaffold was fabricated. However, the scaffold was not easily demoldable, and the number of successfully demoulded hydrogels was lower than with the two previous 3D models, with only one successfully demoulding out of 7 attempts. One of the successfully demoulded patterned scaffold was imaged on the confocal microscope and can be seen in Figure 4.17.

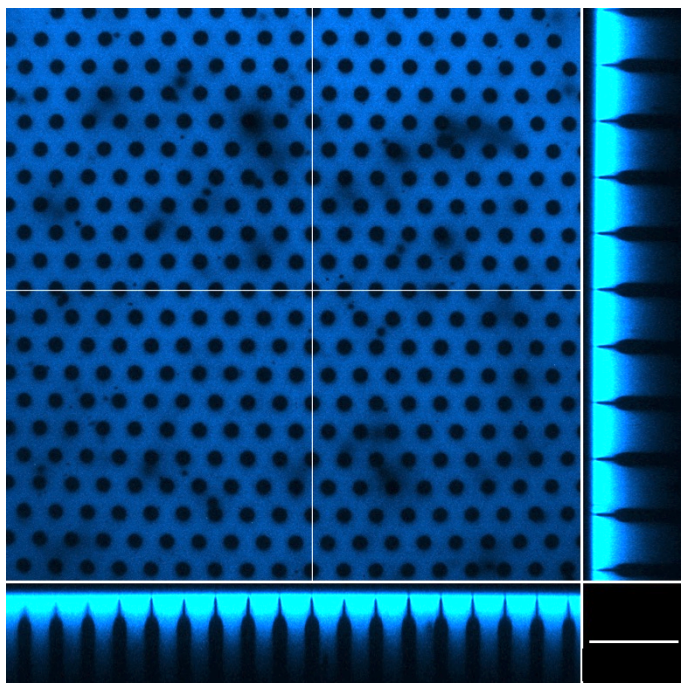


Figure 4.17: Confocal z stack image of a demoulded micro-patterned hydrogel. The demoulding and microscopy were performed by Dr. Eoghan Cunnane. Scale bar 100 μm .

Looking at the image in Figure 4.17, the channels exhibit no excess material on top of the stems. Increasing the print height to 215 μm allowed the tips of the columns on the negative print to be in contact with the cover slide on top. Theoretically having a 200 μm spacer and a 200 μm print would mean that the cover slide would be flush against the top of the print, however practically, the print needed to be taller to allow for this. This outcome showed that it was possible to fabricate patterned scaffolds with channels running through the entire thickness of the hydrogel.

Addressing the issue of demoulding, it was noted that, as the amount of excess hydrogel material on top of the stems decreased with increasing print height, the amount of successfully demoulded hydrogels decreased as well. It was thus theorised that the extra layer of material was useful for demoulding the hydrogel and allowed for extra support and adhesion to the cover slide.

In addition, the unsuccessful demoulding attempts resulted in the hydrogel tearing near the negative print. When looking at these torn hydrogels more closely, it appeared that the tears would occur either around the edge of the entire print, on the outside of wall as seen in figure 4.18, or around the outer edge of the features themselves, as seen in figure 4.19. Therefore, more optimization needed to be performed to resolve the demoulding challenges and will be addressed in the next section.

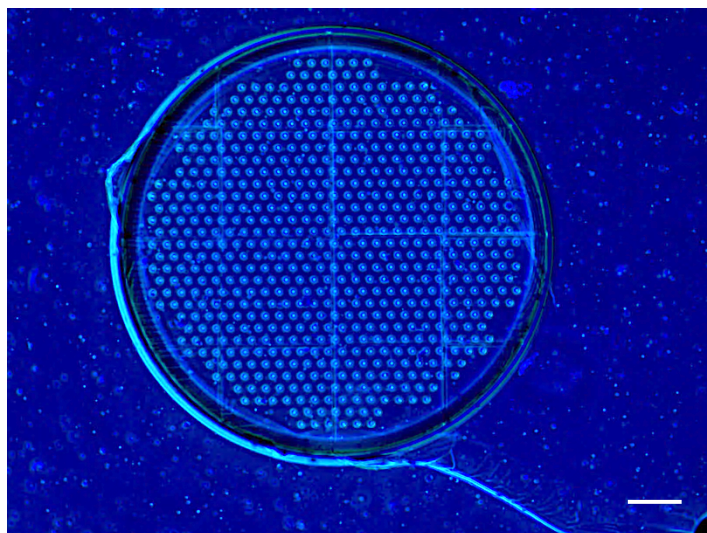


Figure 4.18: Light microscope image taken of a failed demoulded hydrogel. The hydrogel was still stuck to the negative print, using a light microscope. The demoulding and microscopy were performed by Dr. Eoghan Cunnane. Scale bar 100 μm .

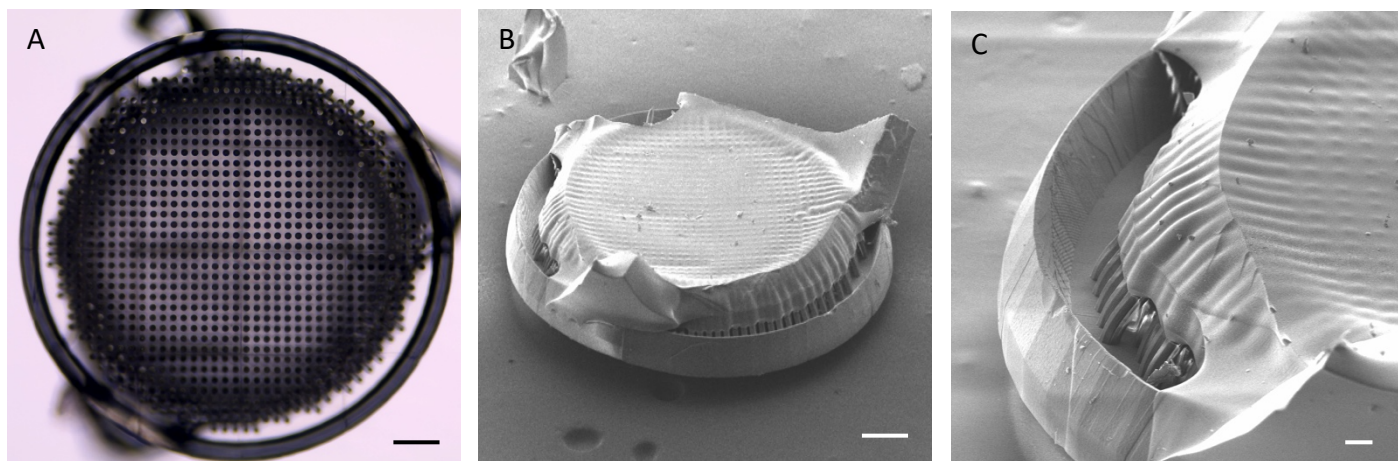


Figure 4.19: Images of a failed demoulded hydrogel still stuck to the negative print. (A) Light microscope image taken of failed demoulded hydrogel still stuck to the negative print, using an EVOS XL core microscope (Thermofisher). (B) and (C) SEM images taken at 60° tilt, at 60X (A) and 250X (D) magnifications, using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bar: (A) and (B): 100 μm , and (C): 20 μm .

4.3 Optimizing the demoulding process:

4.3.1 Changing the print geometry:

To optimize the demoulding process, the print geometry was modified again, with the aim of decreasing the likelihood of the hydrogel tearing. Firstly, the hydrogel tore repeatedly around the outer edge of the printed wall. Looking back at Figure 2.34 in Chapter 2, the outside wall slant was designed with quite a sharp angle (75°), and that slant did not extend all the way to the bottom of the print, only until the base of the columns themselves. This meant that there was a 90° angle formed by the base and the substrate where the scaffold was tearing.

Looking at the literature, this behaviour was not uncommon. Wang *et al.* (2015) studied the von Mises stress during demoulding of nanoimprints [210]. By running finite element analysis and stimulations, they were able to determine the stress concentration locations. In their design, which consisted of square grooves in a PMMA resist, these locations corresponded to the roots and edges of the microstructures, both of which had 90° angles.

Therefore, the wall design was revisited and modified to increase the likelihood of successful demoulding. This can be seen in Figure 4.20, where the cross section of the 3D print is drawn up with the adjusted walls. The angle of the slant was decreased to 40° , and it was extended all the way to the substrate.

The hydrogel was also observed to tear around the outer edge of the columns themselves. Therefore, two modifications were made. The first was to add a fillet edge to the base of each column. It was hypothesised that the extra curvature would decrease the chance of the hydrogel tearing along the sharp edge of the column base. The second modification was based on the assumption that the patterned section could have been slightly too thick ($120\text{ }\mu\text{m}$), with insufficient hydrogel material present in the mould to

prevent tearing during the demoulding. Therefore, the height of the columns was decreased by 25% to 90 μm , while keeping the total height of the print at 215 μm (meaning the base was increased to 125 μm). All the modifications can be seen in Figure 4.20. The adjusted 3D model was printed and imaged on the light microscope and the SEM microscope and can be seen in Figure 4.21.

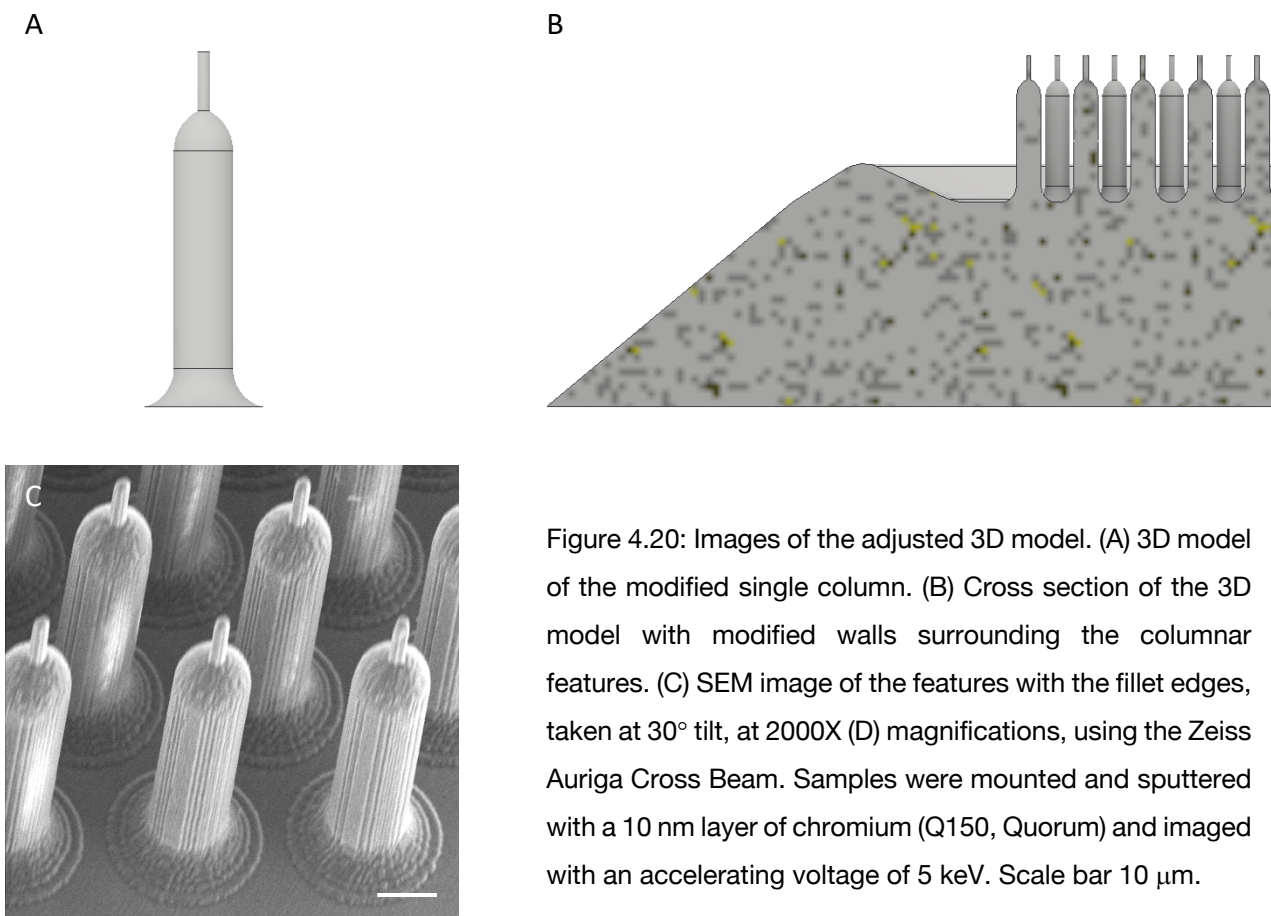


Figure 4.20: Images of the adjusted 3D model. (A) 3D model of the modified single column. (B) Cross section of the 3D model with modified walls surrounding the columnar features. (C) SEM image of the features with the fillet edges, taken at 30° tilt, at 2000X magnification, using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bar 10 μm .

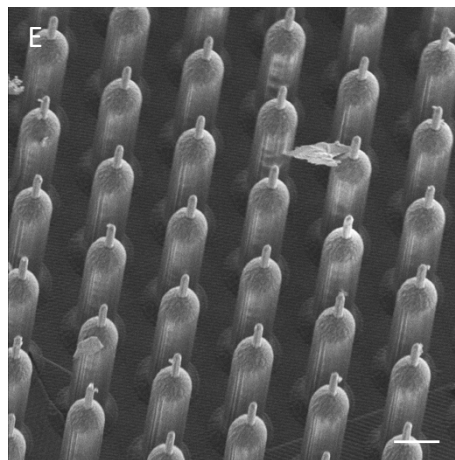
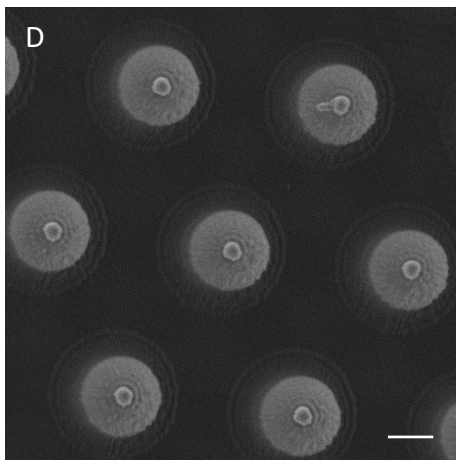
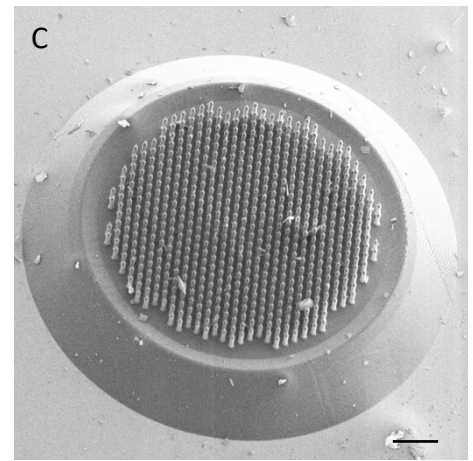
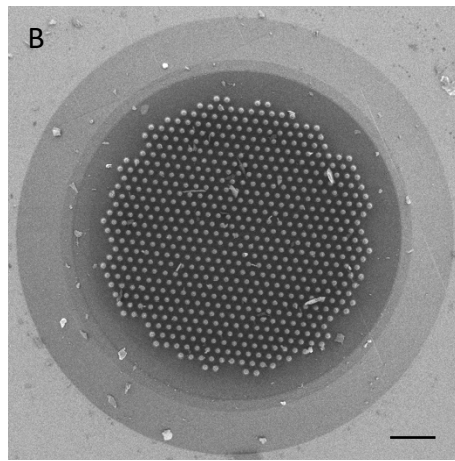
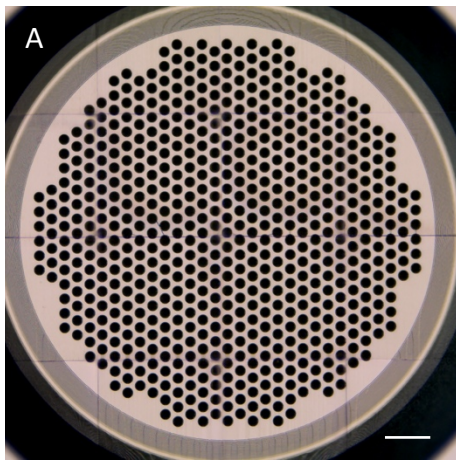


Figure 4.21: Modified print with hexagonal array.

(A): Light microscope image taken after the cleaning step, using an EVOS XL core microscope (Thermofisher).

(B): SEM image taken at 150X magnification.

(C): SEM image taken at 150X magnification and at 30° tilt.

(D): SEM image taken at 2000X magnification.

(E): SEM image taken at 1000X magnification and at 30° tilt.

(F): Top view of 3D model.

(G): Tilted view of 3D model.

(H): Side view of 3D model.

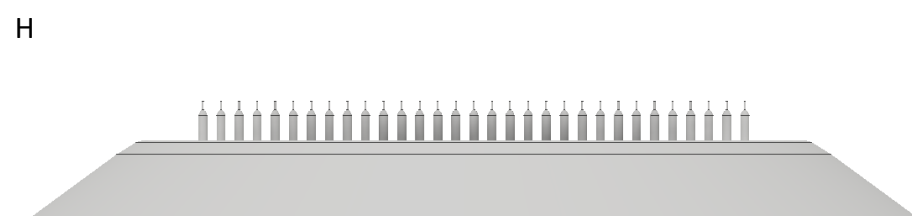
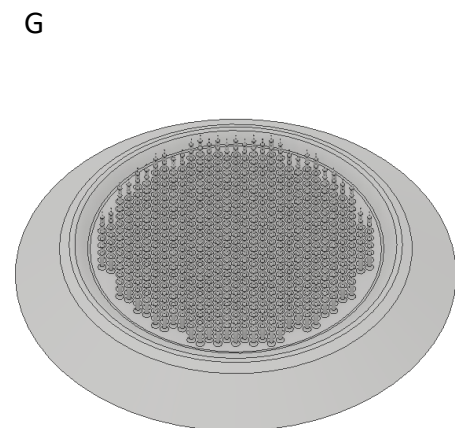
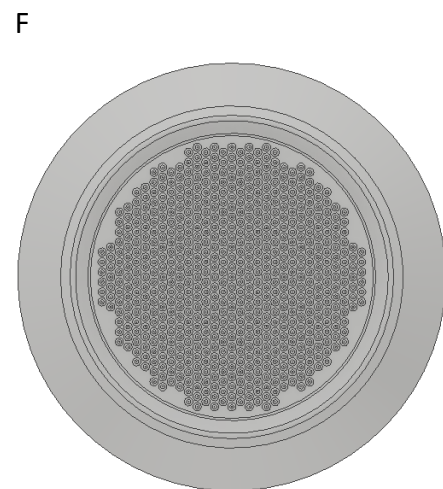
All SEM images were taken using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV.

Scale bars:

(A), (B) and (C): 150 μm

(D): 10 μm

(E): 20 μm



The next step was to attempt to demould a patterned hydrogel from the new printed 3D model. The negative mould was plasma and silane treated to ready the surface for the demoulding step. Using the same 200 μm silicone spacer, and 20 μL of the same gel precursor solution (9% HAMA with Cy5 labelled HAMA added at a ratio of 1:100, 4% PEG and 1% LAP), the hydrogel scaffold was fabricated. However, the scaffold still tore during the demoulding step. The negative print with the scaffold still attached to it was imaged on a light microscope and on a SEM microscope.

Looking at Figure 4.22, there was some progress in the demoulding when compared to the previous 3D model in figure 4.19. With the old model, the scaffold tore around the entire column area, however with the new model, there was some sections that successfully demoulded, and the hydrogel tore around a smaller area of the print.

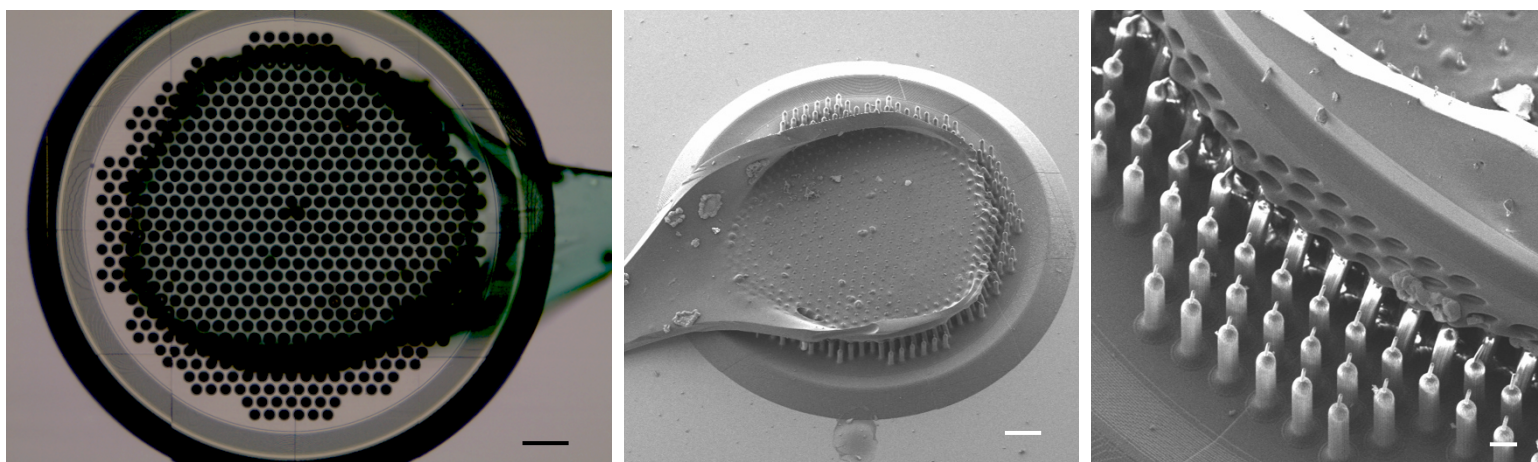


Figure 4.22: Images of a failed demoulded hydrogel still stuck to the negative print. (A) Light microscope image taken of failed demoulded hydrogel still stuck to the negative print, using an EVOS XL core microscope (Thermofisher). (B) and (C) SEM images taken at 60° tilt, at 60X (A) and 250X (D) magnifications, using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bar: (A) and (B): 100 μm , and (C): 20 μm .

4.3.2 Changing the demoulding technique:

In all the previous demoulding attempts, the cover slide was carefully removed by placing a spatula between the cover slide and the spacer and slowly detaching the cover slide, after which it was slowly lifted, and the hydrogel came off with it. Using this method, the hydrogel was demoulded gradually from one side of the negative print to the other. However, as this technique was completely manual, it increased the chances of human error, and may have caused the hydrogel to tear as seen in Figure 4.22 above. These human errors included the possibility of the hydrogel twisting while the cover slip was being detached, as well as the demoulding motion not being continuous.

Additionally, it appeared that the hydrogel began to demould around the outer part of the print but tore as it got closer to the centre, indicating that demoulding was not occurring uniformly across the entire hydrogel. Thus, the demoulding method was modified to be more controlled and reproducible. Instead of removing the cover slide from one end, an approach to detach the cover slide uniformly was developed as seen in Figure 4.23 and consisted of two alterations.

The first was to tape the bottom of the substrate to the bench to fix its position. The remainder of the steps to fabricate the patterned hydrogel were kept the same. After the UV step was completed, more tape was added to the top of the cover slide to form a tape lattice, as shown in Figure 4.23, and the cover slide was removed by pulling the tape up vertically to apply a uniform force to the entire cover slide, and thus the hydrogel would be demoulded evenly. Even though this method still required some manual demoulding, it was more controlled and required less handling, decreasing the chances of ununiform demoulding.

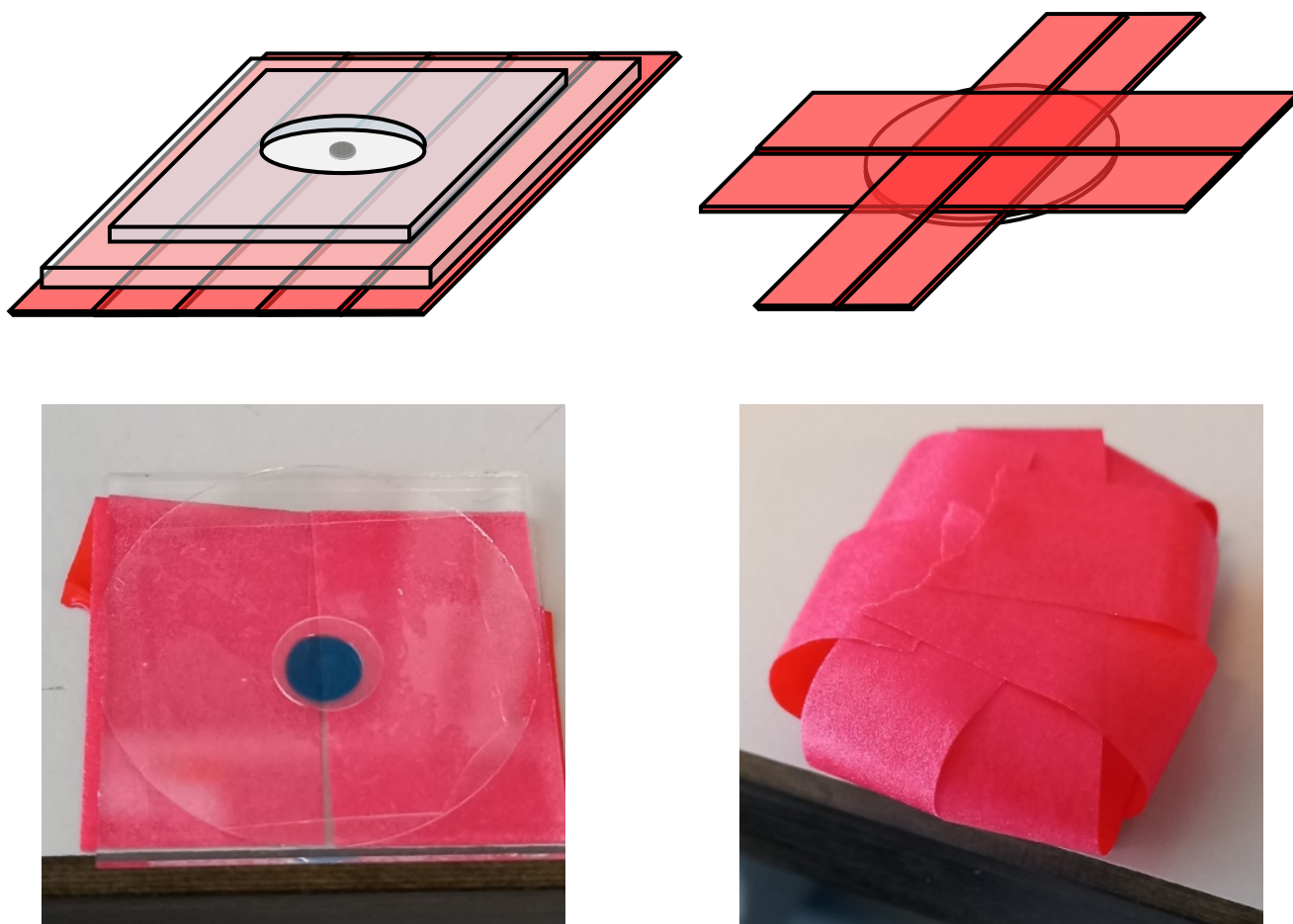


Figure 4.23: Schematics and pictures of the modified demoulding technique. Tape was added to the bottom of the substrate and the bench to fix its position. More tape was added to the top of the cover slide to form a tape lattice, and the cover slide was removed by pulling the tape up vertically to apply a uniform force to the entire cover slide, and thus the hydrogel would be demoulded evenly. Images were taken by Dr. Eoghan Cunnane.

Several hydrogels were successfully demoulded using this method, with no more tearing around the columns or the centre of the print, with 7 out of 10 attempts being successful. One example of a negative print after the hydrogel was demoulded, as well as the hydrogel itself, can be seen in Figure 4.24. Looking at the images, it was clear that this adjusted method was effective in solving the issues previously faced. The negative print remained intact, and only had a very small gel fragment still attached to one of the

columns. Additionally, the patterned hydrogel had perfectly repeating channels, that extended fully through the hydrogel.

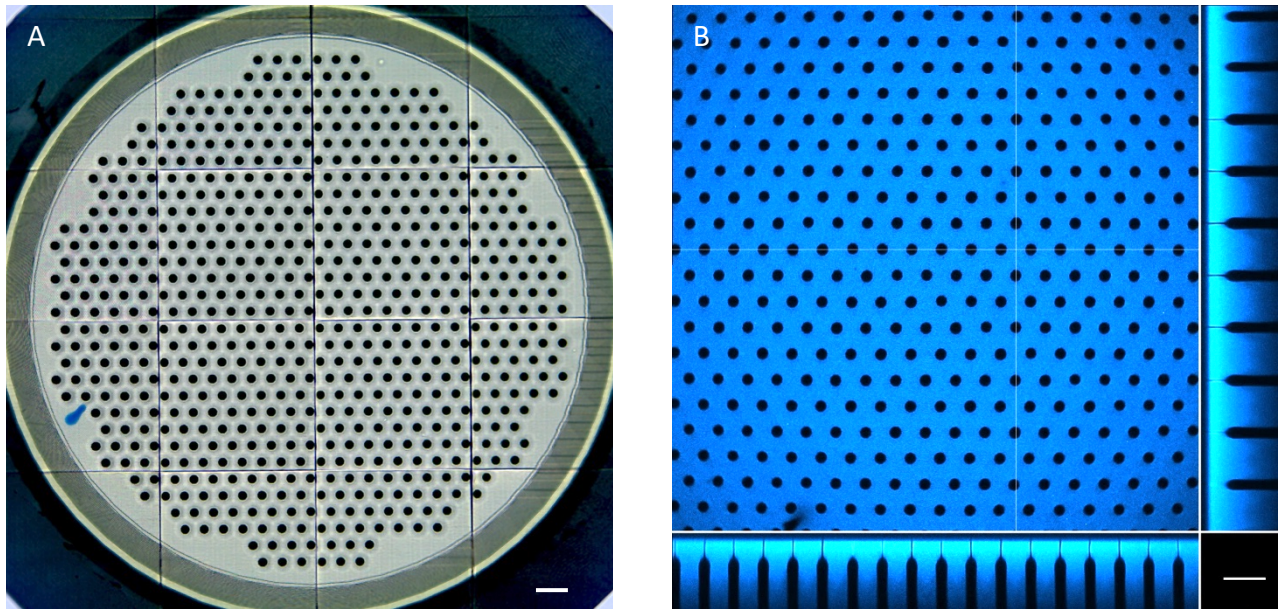


Figure 4.24: Images of a successfully demoulded hydrogel. (A): Light microscope image taken of a negative print after successful demoulding, using an EVOS XL core microscope (Thermofisher). (B): Confocal z stack image of a demoulded micro-patterned hydrogel. The demoulding and confocal microscopy were performed by Dr. Eoghan Cunnane. Scale bar 50 μm .

4.4 Making the process reproducible:

After successfully demoulding patterned hydrogels from the 3D printed moulds, the next step was to make the process as reproducible as possible with the available technology within the lab. Two approaches were attempted to accomplish this and are described in this section.

4.4.1 Final modification to the 3D model:

The adjustments made to the 3D model resulted in the height of the print more than doubling, from 100 μm to 215 μm . With that, the print time of the negative mould also increased from approximately 45 minutes to approximately 100 minutes. This increase meant that less models could be printed in the Nanoscribe time allocated to this project, and it would take longer to optimise or test any new protocols.

Looking back at section 4.2.3, the reason the mould was increased to 215 μm was to adapt to the 200 μm thick spacer used. However, at the time, the height of the individual columns was 100 μm and the thinnest base drawn was 5 μm , meaning that 200 μm was the thinnest available spacer that could be used. Since then, the columns were shortened to 90 μm , meaning that the 100 μm thick spacer could be used instead. Therefore, the total height of the mould was decreased from 215 μm to 115 μm by decreasing the thickness of the base, as can be seen in Figures 4.25 and 4.26, and with that, the print time was decreased by over half to 35 minutes per print. Images (A), (B) and (C) in Figure 4.25 were taken by Thomas Fernandez Debets, a PhD student in the group.

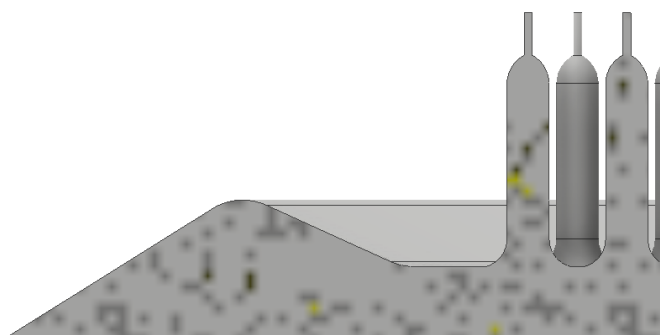


Figure 4.25: Cross section of the 3D model with a total height of 115 μm .

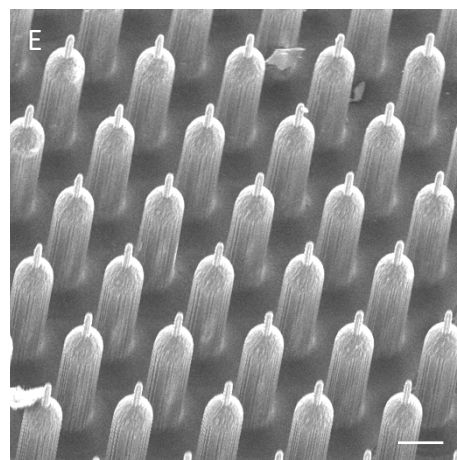
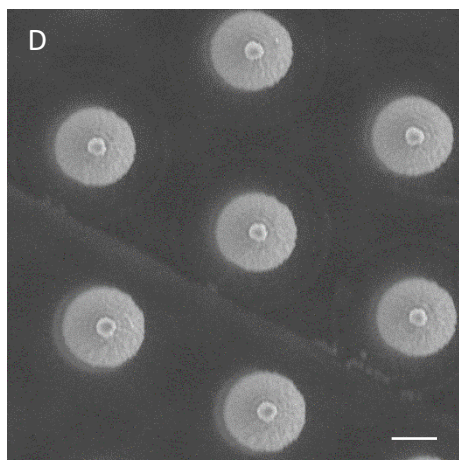
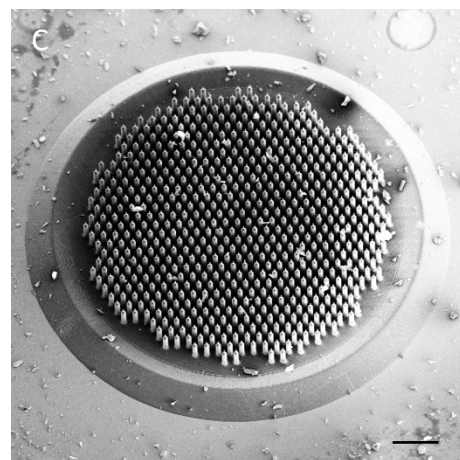
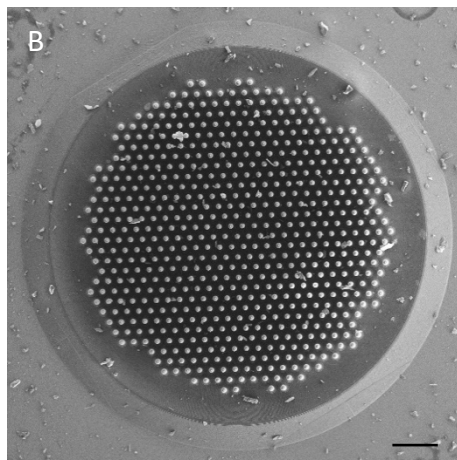
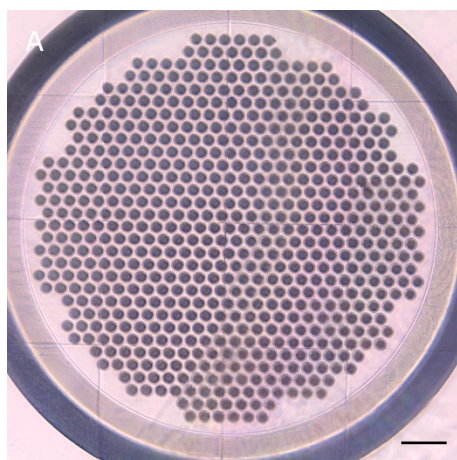


Figure 4.26: Modified print with hexagonal array and 115 μm height.

(A): Light microscope image taken after the cleaning step, using an EVOS XL core microscope (Thermofisher).

(B): SEM image taken at 150X magnification.

(C): SEM image taken at 150X magnification and at 30° tilt.

(D): SEM image taken at 2000X magnification.

(E): SEM image taken at 1000X magnification and at 30° tilt.

(F): Top view of 3D model.

(G): Tilted view of 3D model.

(H): Side view of 3D model.

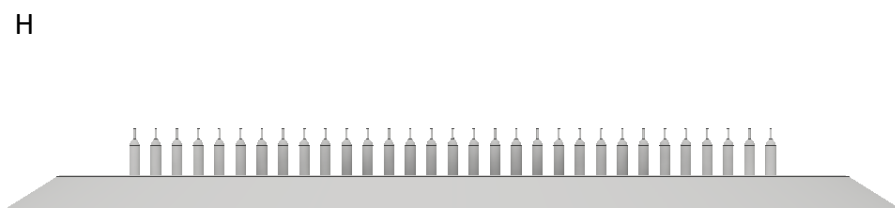
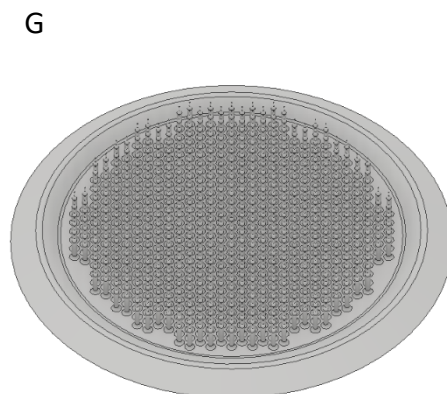
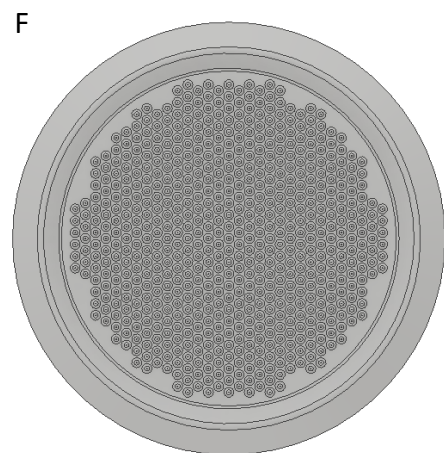
All SEM images were taken using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Images (A), (B) and (C) were taken by Thomas Fernandez Debets.

Scale bars:

(A), (B) and (C): 150 μm

(D): 10 μm

(E): 20 μm



4.4.2 Semi-automatic demoulding setup:

The most challenging part of the entire fabrication protocol was to demould the patterned hydrogel successfully, repeatably, and reproducibly from the negative mould. In section 4.3.2, this step was optimised to a certain extent to decrease the effect of non-vertical motion, as described in that section, however it was still a manual demoulding step, and errors could still occur if the motion was not fully vertical and if there was any twisting motion, both of which would cause the hydrogel to tear.

To make this process semi-automatic, a uniaxial loading machine (ElectroForce 3200, TA Instruments) was used instead to perform a force controlled demoulding step, and the process is pictured in Figure 4.17 below. As this machine did not come with a UV curing setup, one had to be created. A UV light attachment piece was drawn up and 3D printed (orange piece in the setup) to direct the light onto the negative mould/hydrogel setup and allow the gel to polymerise *in situ*.

The protocol consisted of using double sided tape to attach the pre-treated glass substrate with the negative mould to the bottom platform and to attach the plasma-treated cover slide to the bottom of the printed orange holder, which was attached to the top platform (Figure 4.27 (A)). The bottom platform was fixed, and the top platform could be precisely controlled and brought down towards the print, while measuring the displacement, and the detectable force using a load cell.

20 μL of the gel precursor solution (9% HAMA with Cy5 labelled HAMA added at a ratio of 1:100, 4% PEG and 1% LAP) was added to the print, and the top platform was slowly and controllably lowered until the glass slide reached the top of the negative mould (Figure 4.27 (B)): this step will be described in more details shortly. Afterwards, the UV light was switched on at a power of approximately 0.5 mW (S121C, Thorlabs) and left on for 10 minutes to cure the hydrogel in the setup. Finally, the top plate was lifted, and with it the cover slide and the hydrogel attached to it (Figure 4.27 (C)).

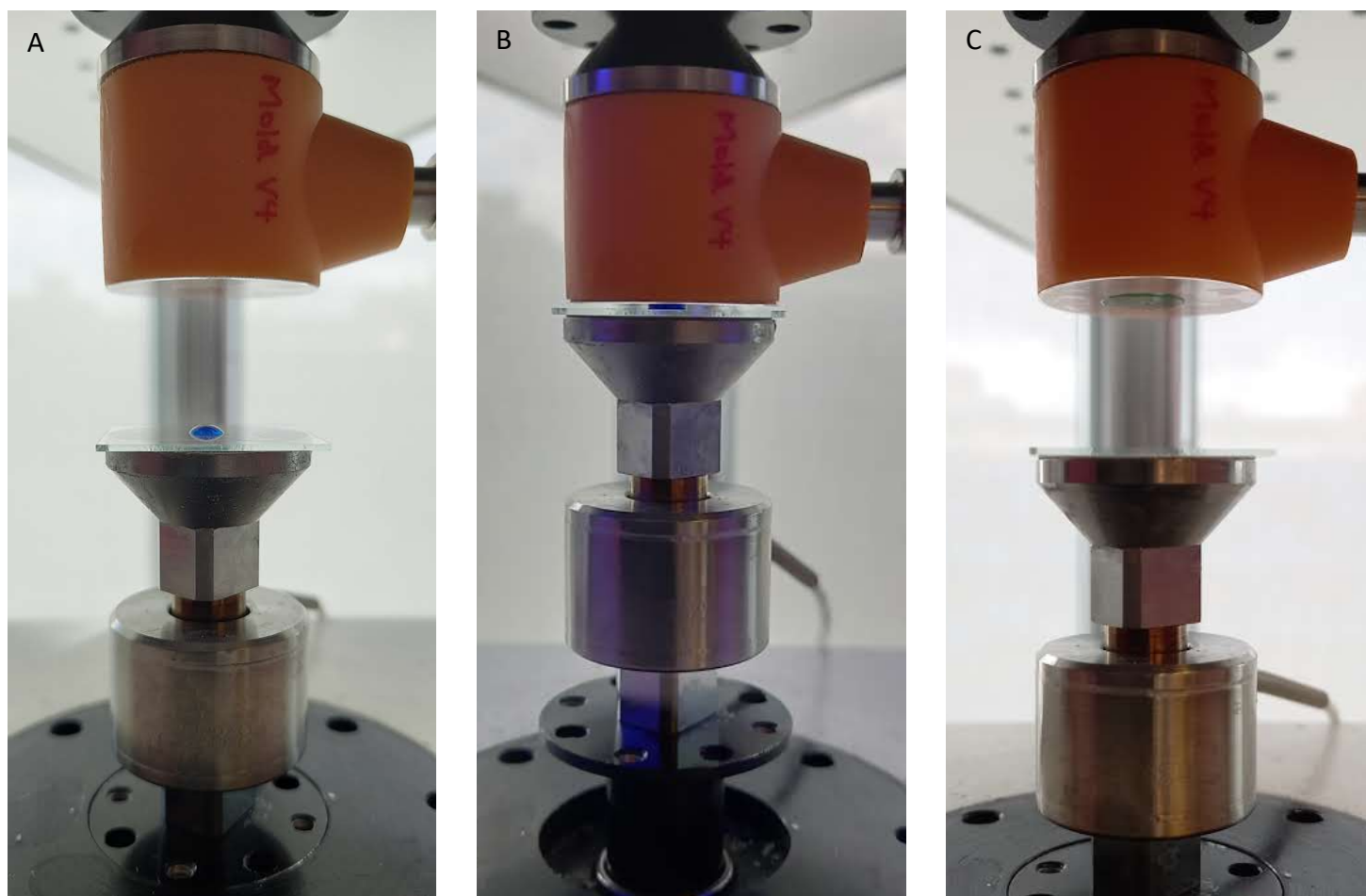


Figure 4.27: Photos of the semi-automated setup on the uniaxial loading machine. (A): The substrate and cover slide were taped to the bottom and top platform respectively, and the hydrogel precursor solution was added to the printed negative. (B): The top platform was lowered until the top of the mould came in contact with the cover slide, and the UV light was switched on. (C): The top platform was lifted with the patterned hydrogel attached to the cover slide. Images were captured by Dr. Eoghan Cunnane and Thomas Fernandez Debets.

In the process described above, the challenge was to identify the point at which the cover slide came in contact with the top of the stems of the negative mould. As the bottom platform was fixed manually, its relative distance to the top platform could not be measured, and therefore distance was not the right parameter to use to determine

the point of contact. Instead, the force reading was used as it is only detected when the cover slide and the mould came in contact.

Therefore, the first step was to determine experimentally the force required to deform the stems of the negative print. A force was applied onto the mould and was gradually increased from 0 to 1 N. As the force increased, the displacement was measured, and the force displacement graphs were plotted for 3 experimental replicates as seen in Figure 4.28.

Looking at the graphs, as the applied force increased, the displacement increased, until the ultimate load was reached. At that point, the mould was plastically deformed, which meant that the applied force caused the stems to bend or buckle. From the graphs, it could be determined that the ultimate load was equal to 0.1673 N.

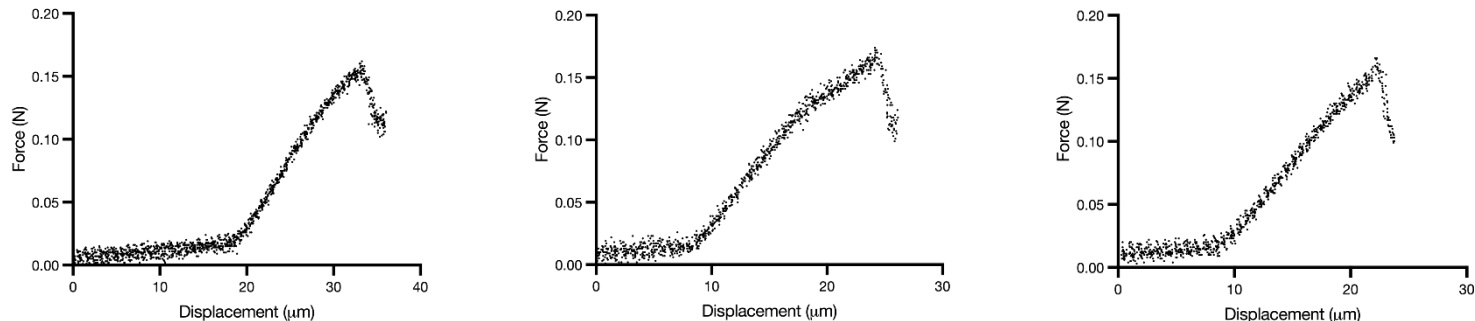


Figure 4.28: Force displacement graphs. The force displacement graphs were plotted for three separate experiments to determine the average force that deforms the stems of the negative print. As the applied force increased, the displacement increased, until the ultimate load was reached. Experiments were performed by Dr. Eoghan Cunnane and Thomas Fernandez Debets.

The force displacement measurements were conducted with the hydrogel precursor solution in the setup, however, to visualise the stem deformation, the same experiment

was repeated with a negative mould on its own, and the resulting negative mould was imaged on the SEM microscope. Looking at the images in Figure 4.29, the deformed stems could be clearly seen, and most of the stems appeared to bend in the same direction.

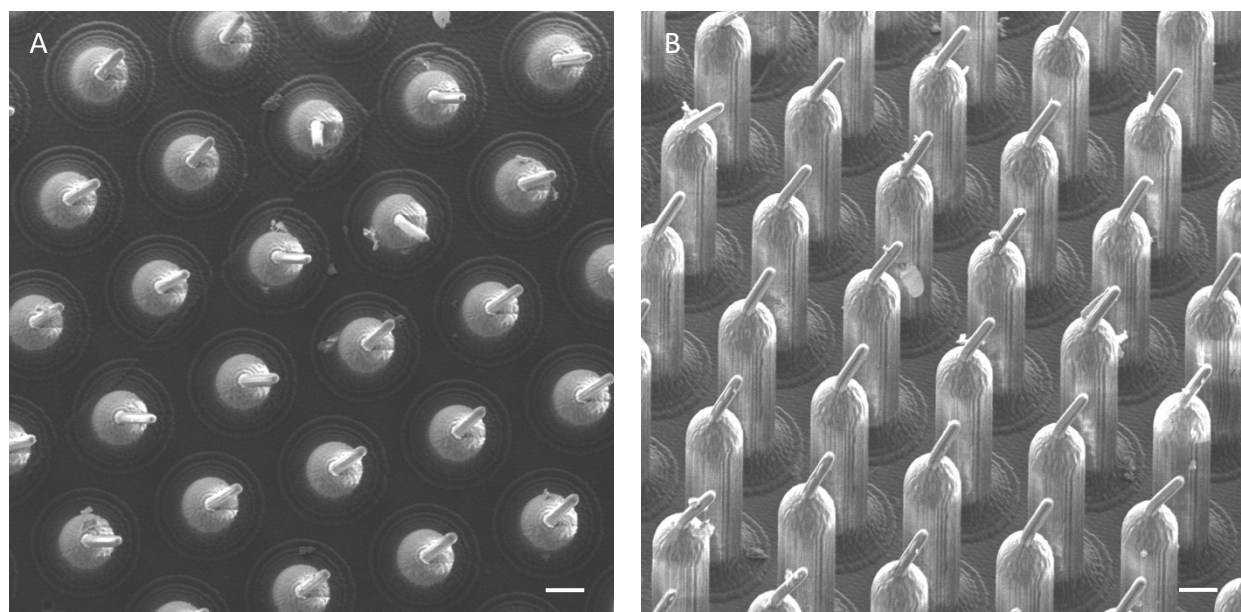


Figure 4.29: SEM images of a negative mould with deformed stems. Images were taken at 500X magnifications, from the top (A) and at a 30° tilt (B), using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bar: 10 μm .

After the force required to deform the negative mould was determined to be 0.1673 N, a force of 0.08 N was chosen to be applied to the setup to still achieve contact between the cover slide and the mould without deforming the stems. Initial tests were conducted without the hydrogel precursor solution to verify that this force would not bend the stems. After the experiment was conducted, the negative mould was imaged on the SEM microscope, from both the top and at a 30° tilt. Looking at the images in Figure 4.30, the stems were unaffected by the applied force and did not undergo any plastic deformation.

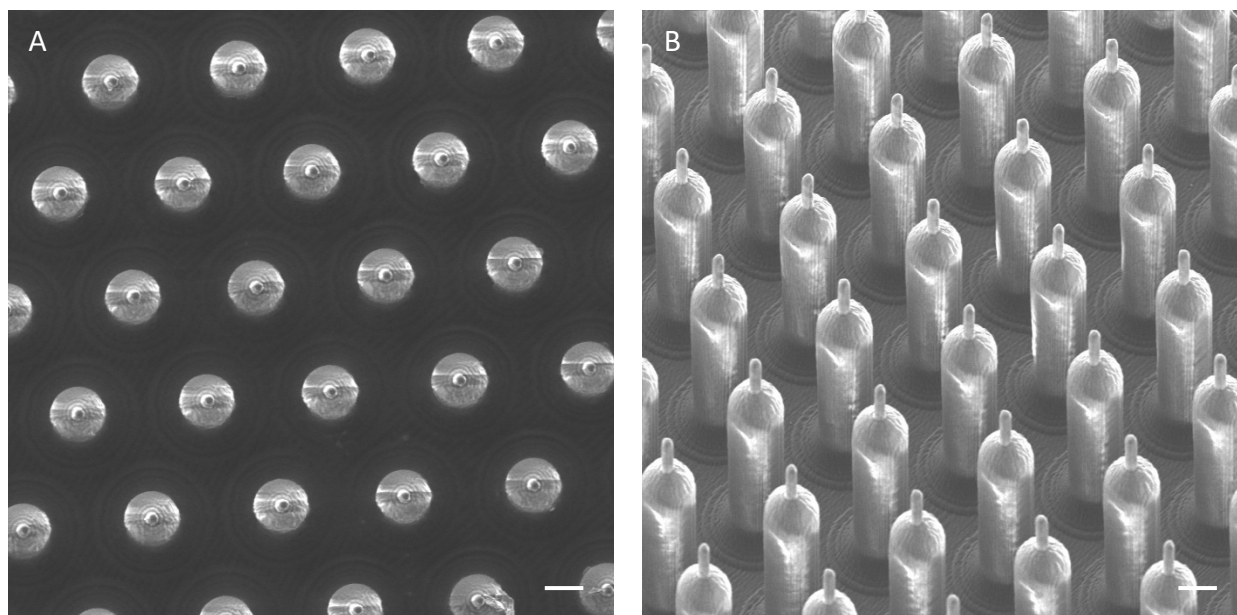


Figure 4.30: SEM images of a negative mould with intact stems. Images were taken at 500X magnifications, from the top (A) and at a 30° tilt (B), using the Zeiss Auriga Cross Beam. Samples were mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. Scale bar: 10 μm .

The final experiments that were conducted were to verify that the chosen force allowed for contact between the cover slide and the mould and produced patterned hydrogels with channels that extended throughout the entire hydrogel. The protocol described previously and illustrated in Figure 4.27 was used for the experiments, with an applied force of 0.08 N. After the gel was cured, the top platform was lifted, and the patterned hydrogel came off the negative mould and remained attached to the cover slide.

To verify that this experiment was successful, the negative mould was imaged on the SEM microscope, and looking at the image in Figure 4.31 (A), the stems were unaffected by the applied force and did not undergo any plastic deformation. Additionally, the patterned hydrogel was imaged on the confocal microscope, and looking at Figure 4.31 (B), it had repeatable channels that extended throughout the entire thickness of the hydrogel (Figure 4.31 (C)).

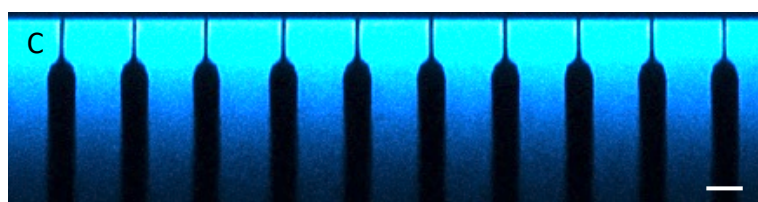
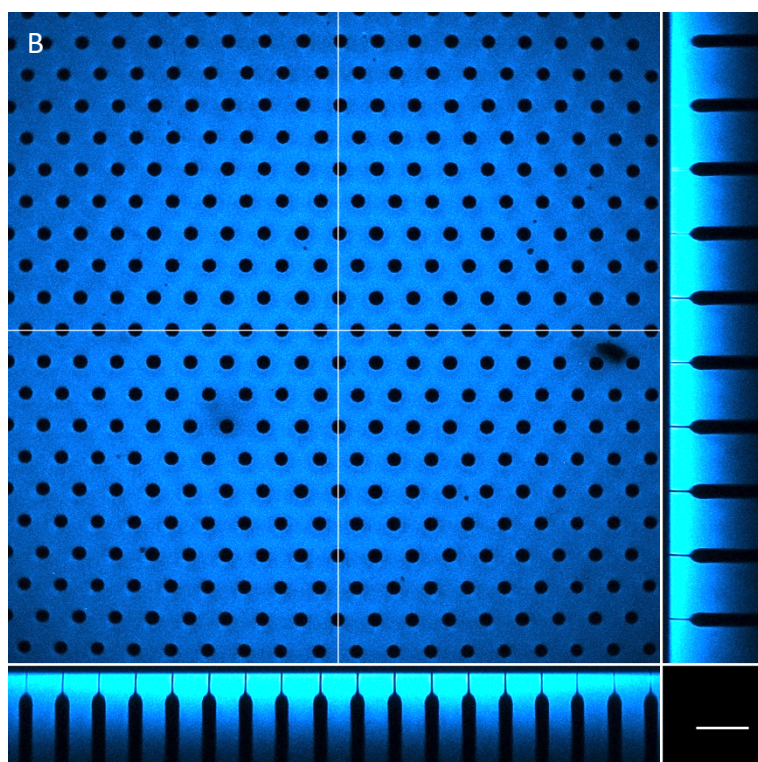
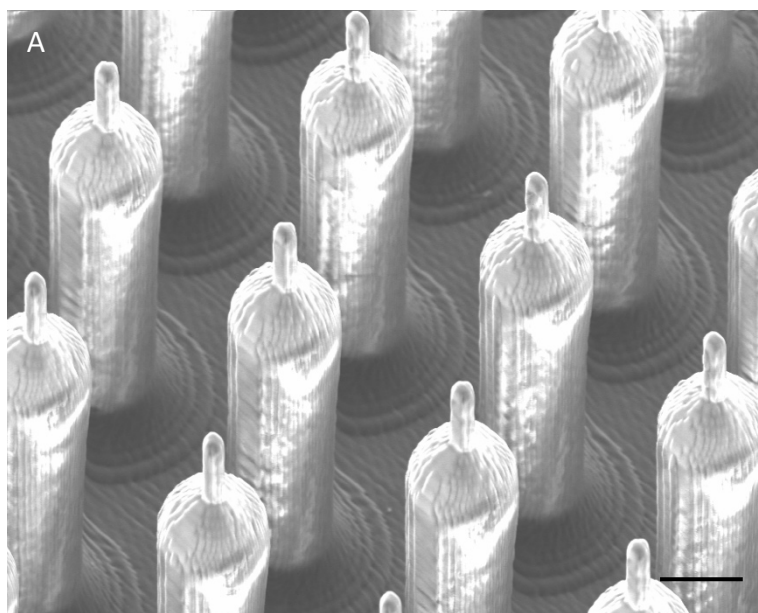


Figure 4.31: Images of a successfully demoulded hydrogel. (A): SEM image of a negative mould with intact stems. Image was taken at 1000X magnification at a 30° tilt, using the Zeiss Auriga Cross Beam. Sample was mounted and sputtered with a 10 nm layer of chromium (Q150, Quorum) and imaged with an accelerating voltage of 5 keV. (B) and (C): Confocal z stack image of a demoulded micro-pattered hydrogel. The demoulding and confocal microscopy were performed by Dr. Eoghan Cunnane and Thomas Fernandez Debets. Scale bar: (A): 10 μm , (B): 50 μm , (C): 20 μm . The figure can be seen in the page above.

4.5 Conclusion

Extensive optimisation of the scaffold fabrication process was performed to successfully and repeatably produce the final scaffold in a semi-automated manner. The first experiments focused on producing a scaffold with the desired pattern, and more specifically, with the designed channel geometry and no excess hydrogel material. Once that was successful, the demoulding process was optimised, and the setup was adapted to a uniaxial loading machine to perform a force-controlled demoulding. This resulted in a patterned hydrogel with repeating channels with no excess material, and a negative mould with no damage.

Chapter 5: Primary photoreceptor cells culture experiments: cell isolation protocol and preliminary studies

The remaining experiments conducted in this project were cell culture experiments. This chapter provides the primary photoreceptor cells isolation protocol, as well as preliminary cell culture experiments. These cells were cultured on top of flat hydrogels, using both GelMA and HAMA with PEGDMA, and cultured in pre-incubated media, to test the cytotoxicity of the hydrogel formulations on the cells. The results of this chapter were obtained in collaboration with Dr. Aikaterini Kalargyrou.

5.1 Protocol for dissociating and sorting photoreceptors

The protocol for dissociating and sorting photoreceptors was developed and optimized by Dr. Aikaterini Kalargyrou, a postdoctoral researcher in the Pearson group collaborating on this project [211].

5.1.1 Mouse lines

The preliminary cell culture experiments were conducted using photoreceptors from a Nrl.GFP^{+/+} mouse line [49]. As discussed in Chapter 1, the neural retina leucine zipper transcription factor is an essential factor in rod photoreceptor differentiation and was used to drive the expression of EGFP in the rod photoreceptors of the mouse line. The mice used in this chapter were sacrificed at developmental stage ranging from P4-P8 **MT** through cervical dislocation, based on the results from Pearson *et al.* 2012, which showed that cells collected from this developmental stage window achieved the best outcome when transplanted [47].

All the mice were maintained and sacrificed in the animal facilities at University College London (until March 2020) and at Kings College London (from May 2021). All work was

carried out in accordance with the UK regulations and legislation, more specifically the Animals (Scientific Procedures) Act 1986 of the UK government.

5.1.2 Retinal dissection

The retinal dissection was performed using a modified version of the protocol described in Pearson *et al.* 2012. Briefly, after the animals were sacrificed, the eyes were collected and placed in a solution of freshly made dissection medium. The dissection medium consisted of a buffer (can be any of the following, all with no phenol red: DMEM/F12, PBS, HBSS, and EBSS), 15 mM of HEPES, 1 mM of Taurine and 0.5 unit/mL of DNase (Desoxyribonuclease I). To dissect the retina from the rest of the collected eye, a small puncture was first introduced in the eye to allow for liquid exchange. Next, forceps were used to create a small hole in the corneal region of the eye to remove the cornea entirely. A small cut was then made in the sclera, and the retina was separated from the choroid and sclera using two pairs of forceps. The dissociated retinas were transferred into 15 mL falcon tubes and placed 10 at a time in 5 mL of the dissection buffer per tube. Finally, the isolated retinas were washed twice with 5 mL of the dissection buffer and were ready for the dissociation step.

5.1.3 Prepping the culture area

Before starting the dissociation, the photoreceptor culture surface, which was either tissue culture plastic or the surface of the hydrogel, was prepped and coated to allow the cultured photoreceptors to better attach to the surface. First, the surfaces were coated with Poly-D-Lysine (PDL) by adding 100 μ L of the PDL solution to each well. The PDL stock solution was prepped by first reconstituting the PDL-HBr powder (poly-D-lysine hydrobromide, Sigma Aldrich) in 50 mL of sterile distilled water at a concentration of 5 mg/mL, and then diluted 1 to 5 in sterile distilled water to make a working solution. The solution was left to bind for 10 minutes at room temperature in the hood. Following that, the solution was aspirated, and the wells were left to air dry for 10 minutes in the

hood. Once completed, the wells were washed once with sterile distilled water. The PDL coating facilitates the attachment of the second coating to the surface.

The second coating was a fibronectin matrix, a known ECM component that cells can adhere to. The fibronectin solution was created by reconstituting the fibronectin powder (Fibronectin, Sigma Aldrich) in PBS at a concentration of 2 mg per 50 mL, and then 100 μ L of the solution was added to each well. The plate was incubated at 37 °C for anywhere between 1 to 19 hours. Once the cells were ready to be cultured, the solution was aspirated. Both coating solutions were stored at 4 °C for one month, after which they were discarded.

5.1.4 Retinal dissociation

5.1.4.1 Dissociation kit and its components

The retinal dissociation was conducted using a Papain Dissociation System, that was purchased from Worthington Biochemical Corporation (<https://www.worthington-biochem.com/PDS/>). This dissociation kit is based on the Huettnner and Baughman method to dissociate neurons from the visual cortex of postnatal rats and yield viable and morphologically intact neurons [212]. This kit uses papain as a proteolytic enzyme as it has been shown to be less damaging and more effective compared to other proteases and has been previously used to dissociate retinal cells [73].

The kit came with a buffer, the papain enzyme, DNase, and OMI. The buffer consisted of sterile EBSS (Earle's Balanced Salt Solution) supplemented with calcium, magnesium, bicarbonate, and phenol red. The DNase was reconstituted with 0.6 mL of buffer to make a solution at a concentration of 2000 unit/mL, and then aliquoted into 50 μ L vials that were stored in the -20 °C freezer for up to 2 months. The OMI, or ovomucoid protease inhibitor with bovine serum albumin, was reconstituted with 32 mL of the buffer to make a solution containing 10 mg/mL of both the ovomucoid inhibitor and the albumin, that

was stored in the fridge at 4 °C for up to 3 months. Lastly, the papain solution, containing L-cysteine and EDTA (Ethylenediaminetetraacetic acid), was reconstituted with 5.5 mL of the buffer to make a solution at a concentration of 20 unit/mL of papain, with 1 mM L-cystein and 0.5 mM of EDTA. Additionally, the papain solution was supplemented with 250 µL of the reconstituted DNase solution to make the concentration of DNase 0.005%. The solution was stored in the fridge at 4 °C for up to 2 weeks.

5.1.4.2 Enzymatic digestion of the retinal tissue

The papain solution was first placed in the water bath at 37 °C for 10 minutes to activate the enzyme. Afterwards, 3 to 4 retinae were transferred into 500 µL of the papain solution in a 15 mL falcon tube, gently triturated 2 to 3 times using a wide bore P1000 tip and incubated for at least 10 minutes in the water bath. During that time, the tubes were gently agitated every 5 minutes to facilitate the dissociation of the tissue into single cells. Depending on the developmental stage of the mice the retinas were collected from, the retinal tissue required slightly different time to dissociate.

5.1.4.3 Inhibition of papain enzyme

After the incubation was completed, the cell mixture was triturated with a regular P1000 tip about 10 times. The dissociated cell solution was strained into a round bottom tube with a cell strainer cap (a different tube and strainer was used for each 1 mL of solution), and then the tubes were combined and centrifuged at 260 x g for 5 minutes at room temperature.

Following this, the supernatant was discarded, and the cell pellet was reconstituted in resuspension buffer, by adding 200 µL of buffer per 5 retinae. The resuspension buffer consisted of 2.7 mL of EBSS, 300 µL of the OMI solution and 50 µL of the DNase solution. A solution of the OMI was added to a new centrifuge tube, at a volume equivalent to 700 µL of OMI per 5 retinae. The cell suspension was then layered over the OMI solution by

pipetting the solution slowly on the side of the tube, to form a discontinuous gradient. The tube was centrifuged at 70 x g for 10 minutes at room temperature. This process formed a gradient with two layers and an interface clearly visible between them. In this gradient, the dissociated cells were pelleted at the bottom of the tube, while the membrane fragments remained at the interface level.

5.1.4.4 Resuspension of the cell pellet

After the gradient was formed, the supernatant was discarded, and the pellet was reconstituted in 1 mL of buffer for magnetic-activated cell sorting (MACS). The MACS buffer consisted of PBS without Ca/Mg, 15 mM of HEPES, 1% BSA, and DNase at a concentration of 0.005%.

5.1.5 Photoreceptor sorting and isolation

5.1.5.1 MACS

Magnetic-activated cell sorting, or MACS, is a method of cell isolation. It works by first binding magnetic beads or particles to the target cells through antibody interactions with surface markers on the cells. Then, the cells are transferred to a column, and exposed to a magnetic field, to temporarily magnetically bind the cells to the column while the non labelled cells flow through. Finally, the column is removed from the magnet and the labelled target cells are collected.

The magnetic-activated cell sorting kit was purchased from MiltenyiBiotec, in addition to the columns and the separator. LS columns were chosen as they were designed for positive selection of cells. The columns are composed of ferromagnetic spheres with a cell friendly coating that, when placed in the magnetic field of the MACS separator, amplify the field strength 10,000-fold, and allow for isolation of cells bound to a magnetic bead, while still allowing non-labelled cells to flow freely through the column.

MACS was chosen instead of FACS (fluorescence-activated cell sorting) because it produces a higher yield and less cell loss. In a paper published by Sutermeister *et al.* 2019, the authors showed that MACS resulted in only 7 to 9% cell loss, compared to about 70% cell loss due to FACS. Additionally, MACS was up to 6 times faster than FACS [213].

5.1.5.2 Attaching the magnetic beads to the cells

To calculate the volume of primary anti-mouse-CD73 APC-conjugated antibody solution to add to the photoreceptors, the number of cells needed to be determined. To count the number of cells in the suspension, 50 μL of the solution was added to 450 μL of MACS buffer for the cell count. The remaining cell suspension was centrifuged again at 70 x g for 10 minutes at room temperature, and the supernatant was discarded. The pellet was reconstituted in MACS buffer, at a concentration of 10^6 cells per 100 μL . The primary anti-mouse-CD73 APC-conjugated antibody was also added at a concentration of 1 μL per 10^6 cells. The solution was left in the fridge at 4 °C for 30 minutes, and the tube was gently agitated halfway through.

Once completed, 5 mL of the MACS buffer was added to the cells to wash them, and the solution was centrifuged at 260 x g for 10 minutes at 4 °C to get rid of any unbound antibodies. The supernatant was discarded, and cell pellet was resuspended in the anti-APC solution at a concentration of 20 μL of anti-APC-Microbeads and 80 μL of dissection buffer per 10^7 cells. The solution was left in the fridge at 4 °C for 30 minutes, and the tube was gently agitated halfway through.

5.1.5.3 Manual MACS sorting

The MACS was setup by placing the magnetic separator as well as the column in the hood, and the column was washed with 1 mL of MACS buffer. Then, depending on the volume of the solution, the cells were resuspended to a total volume of 0.5 mL of buffer.

The sample was loaded into the column, and an additional 3 mL of MACS buffer was added on top. The eluate, which corresponded to the negative fraction of cells not bound to the magnetic beads, was collected into a 15 mL falcon tube. The column was washed with 3 mL of buffer to flush out any remaining unbound cells.

To collect the positively labelled photoreceptors, the column was removed from the magnetic separator and placed into a new falcon tube to collect the cells. An extra 2 mL of buffer was added to the column, and the plunger was placed to introduce some pressure and force the cells down the column. The flowing solution should have a light brown color, which indicates the cells in the solution are the CD73 positive fraction bound to the magnetic beads.

The cells needed to be resuspended in photoreceptor media, therefore the collected solution was then centrifuged at $260 \times g$ for 10 minutes at room temperature, and the supernatant was discarded. The pellet was resuspended in 1 mL of media, and 50 μL was removed and added to 450 μL of media for the cell count.

The photoreceptor chemically defined media (PCDM) consisted of BrainPhys™ Neuronal Medium with no phenol red, 15 mM of HEPES, 1 mM of taurine, 1 to 3% BSA, 100X ITS (Insulin-Transferrin-Selenium) diluted to 1X, and antibiotics Pen/Strep at 10 mg/mL. The media was stored in the fridge at 4 °C for up to 2 weeks.

5.2 Photoreceptor cultures on top of flat gels

Experiments were carried out with an un-patterned material to determine the biocompatibility of the chosen material with the photoreceptor cells. These experiments were performed to identify the optimal concentrations for the photoreceptor cells to adhere to the hydrogel and form processes. These tests were conducted on flat gels with different materials and concentrations and will be described in this section.

5.2.1 Preliminary GelMA testing

To examine the effect of GelMA concentration on photoreceptors, 90 and 180 mg/mL were tested. The addition of 90 mg/mL of PEGDMA to 90 mg/mL of GelMA was also tested and compared to PEGDMA on its own. These conditions were also compared to a tissue culture plastic control and were coated in the same way with PDL and fibronectin.

5.2.1.1 Materials and methods

5.2.1.1.1 Preparing the hydrogel in the plate

The gel precursor solution was prepared by weighing the lyophilised GelMA and/or PEGDMA into an Eppendorf tube and adding sterile 1X PBS to dissolve it. The solution was then placed in an oven at 60°C for an hour to fully dissolve the GelMA and/or PEGDMA, to decrease the viscosity of the solution and to make the solution more handleable. Following this, LAP was added at a concentration of 0.5 mg/mL, the tube was kept covered in aluminium foil to prevent any polymerisation from the surrounding light, and then vortexed to dissolve the LAP.

The hydrogels were fabricated in a μ -Plate Angiogenesis 96 well plate (Ibidi) by pipetting 10 μ L of the appropriate gel precursor solution into the inner chamber of each well in a sterile environment. Following this, the plate was centrifuged at 100 x g for 2 minutes and placed under a UV lamp to crosslink the gel in the plate. Finally, the gels were washed 3 times with PBS and 3 times with DMEM/F-12 cell culture media to remove any unbound gel materials still adhered to the surface of the gel. The plate was left overnight in the incubator in DMEM/F-12 to allow them to facilitate solvent exchange with the gels.

This Ibidi plate was chosen for the initial testing for several reasons. Firstly, the ‘well-in-a-well’ geometry allowed for fabrication of flat gels with no meniscus. Secondly, the geometry also allowed for a reduction of the volume of gel precursor to be used per well.

5.2.1.1.2 Photoreceptor culture protocol

The photoreceptors were dissociated according to the protocol described in section 5.1. Three different concentrations of cells were examined for this experiment to determine the optimal number of cells to culture per well to achieve a high cell viability, while still plating the cells sparsely enough to be able to visualise process formation: 100,000 cells, 125,000 cells, and 150,000 cells per well. These numbers were chosen based on previous experiments carried out in the Pearson group.

The cells were seeded on top of gels and incubated for 72 hours at 37 °C and 5% CO₂. Experiments were conducted in triplicate. Once the incubation period had passed, the cells were imaged on Zeiss Axio Observer Z1 microscope to visualise their distribution and viability. As the cells were dissociated from retinæ collected from Nrl.GFP^{+/+} mice, they were fluorescent, and could be imaged without being fixed and stained.

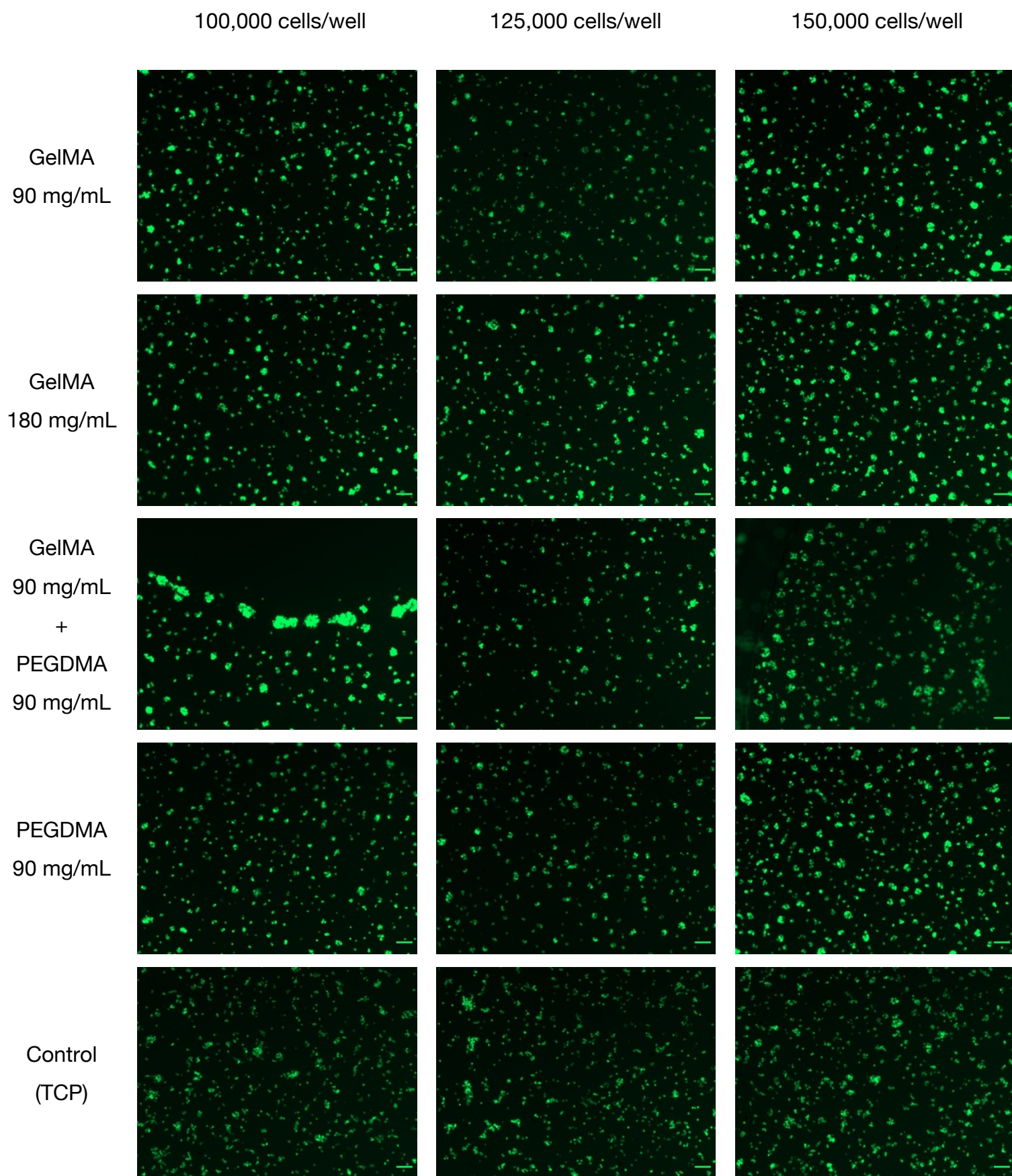
Once the images were acquired, the cells were detached from the surface of the hydrogel or tissue culture plastic using a 2.5% solution of Trypsin-EDTA to obtain a quantitative measurement of viability. Briefly, the media was aspirated, and the cells were washed with PBS. Afterwards, 20 µL of the trypsin solution was added to each well and the plate was incubated for 10 minutes to activate the enzyme. Once completed, 50 µL of media was added to the wells to inhibit further action of the trypsin. Finally, the cells were collected, and the replicates were pooled together by condition to quantify the number of viable cells. An automated cell counter was used (Vi-CELL XR Cell Viability Analyzer, Beckman Coulter Life Sciences).

5.2.1.2 Results and discussion

Microscopy images of all the wells were acquired, however only one representative image per condition can be seen in Figure 5.1. The cells appear to cluster on all hydrogel conditions more than on the tissue culture plastic controls. However, the cells appear viable as seen by their fluorescence. Nevertheless, at this magnification, it was not clear if the cells had developed any processes.

Looking at the graphs in Figure 5.2, it appeared that the viability of the cells on all conditions was above 90% and comparable to the viability of cells on tissue culture plastic. However, the total number of viable cells was different between conditions, with 530,000 cells on all tissue culture plastic conditions, and 460,000 and 480,000 cells on the 90 and 180 mg/mL GelMA conditions, respectively. The conditions with PEGDMA had lower number of viable cells, closer to 300,000 cells.

Figure 5.1: Microscope images of the photoreceptor cells seeded on GelMA. The photoreceptors were seeded on various hydrogel concentrations and at different densities, imaged at 10X. Scale bar 500 μm . The figure can be seen on the next page.



Even though the viability was high for all material concentrations tested, the more relevant data was the number of viable cells. This is because, after 72 hours of incubation, the dead cells floated in the media and removed during the washes. The remaining cells that were attached were the live cells, and they were the ones collected, explaining their high viability percentages.

From this initial data, it appeared that a higher number of cells were attached to the GelMA than to the gels containing the PEGDMA. However, as outlined in Chapter 2, GelMA was no longer the material of choice for this project, cell culture experiments using this biomaterial were therefore not pursued further. The results therefore represent N=1, and no statistical analysis could be performed.

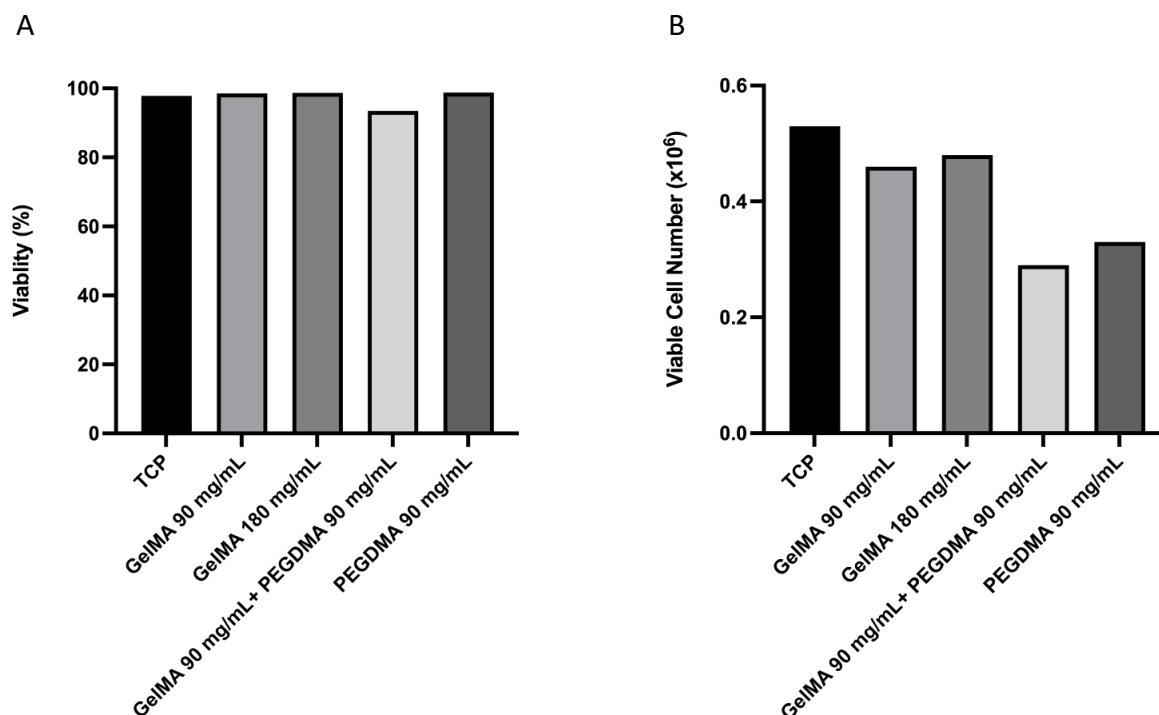


Figure 5.2: Viability measurements of photoreceptor cells seeded on GelMA. Measured viability percentage (A) and number of viable cells (B) plotted for each of the biomaterial conditions tested (N=1).

5.2.2 HAMA testing and optimization

To determine which concentrations of HAMA and PEGDMA were the most suitable for supporting photoreceptors cells, experiments were carried out with un-patterned hydrogels to determine the biocompatibility of these cells on the hydrogels. First, the coating for the hydrogels was tested on just the 9% HAMA – 4% PEGDMA concentration, as that was the most promising combination from the material characterisation experiments. Following that, all of the 25 conditions previously characterised in Chapter 3 were tested and were compared to a tissue culture plastic control coated with PDL and fibronectin. The materials and methods were the same for both experiments conducted.

5.2.2.1 Materials and methods

5.2.2.1.1 Preparing the hydrogel in the plate

The gel precursor solution was prepared by weighing lyophilized HAMA, PEGDMA and LAP into separate Eppendorf tubes, corresponding to the concentrations tested. Sterile 1X PBS was added to the LAP, and that solution was added to the PEG and vortexed until the PEG was fully dissolved. Following that, the new solution mixture was pipetted on top of the HAMA and left overnight at 4°C to dissolve the lyophilised HAMA. The tubes were kept covered in aluminium foil to prevent any polymerisation from the surrounding light.

The hydrogels were fabricated in the same μ -Plate Angiogenesis 96 well plate (Ibidi) by pipetting 10 μ L of the appropriate gel precursor solution into the inner chamber of each well in a sterile environment. Following this, the plate was centrifuged at 100 x g for 2 minutes and placed under a UV lamp to crosslink the gel in the plate. Then, the gels were washed 3 times with PBS, and left for an hour under the UV lamp to sterilise the hydrogels and the wells. Finally, the gels were washed 3 times with photoreceptor media to remove any unbound gel materials still adhered to the surface of the gel. The plate

was left overnight in the incubator in photoreceptor media to facilitate solvent exchange withing the gels.

5.2.2.1.2 Photoreceptor culture protocol

The photoreceptors were dissociated according to the protocol described in section 5.1. The cells were seeded on top of the gels at a concentration of 2 million cells/mL and incubated for 48 hours at 37 °C and 5% CO₂. Experiments were conducted in triplicate. Once the incubation period had passed, 20 µL of the cell proliferation assay MTS reagent was added to each well, and the plate was incubated for 4 hours at 37 °C and 5% CO₂ (the plate could be incubated for 30 minutes to 4 hours). The plate was placed on a shaker for 1 minute, following which the absorbance of the samples was measured on a plate reader at 490 nm against a blank control.

5.2.2.2 Results and discussion

5.2.2.2.1 Testing the coatings

The first experiment was intended to test whether a coating was required on the hydrogels for the photoreceptor cells to adhere to the surface and grow processes. Therefore, cell viability and morphology were assessed on 9% HAMA – 4% PEGDMA hydrogels and compared to 2D cultures on tissue culture plastic coated with fibronectin. *In vivo*, photoreceptor processes protrude to form synaptic connections with surrounding bipolar and horizontal cells. In 2D cultures, the cells extend protrusions to sense the surrounding culture environment and search for cell binding proteins in the surface coating. If the processes cannot form any connections, they retract, usually within 24 hours. Therefore, processes formation and axon pathfinding can be linked to adhesion and is commonly used as a reference for healthy neuronal cells [211], [214], [215].

The gels were tested in triplicate, and the viability was determined from the absorbance measurements of the MTS assay after the cells were cultured for 48 hours. Additionally, the cells were imaged, and the length of their processes was measured manually on ImageJ to compare the morphologies of the cells on the hydrogels to the fibronectin coated tissue culture plastic control. The values for viability and processes lengths were plotted in Figure 5.3, and representative images of the cells can also be seen in this Figure.

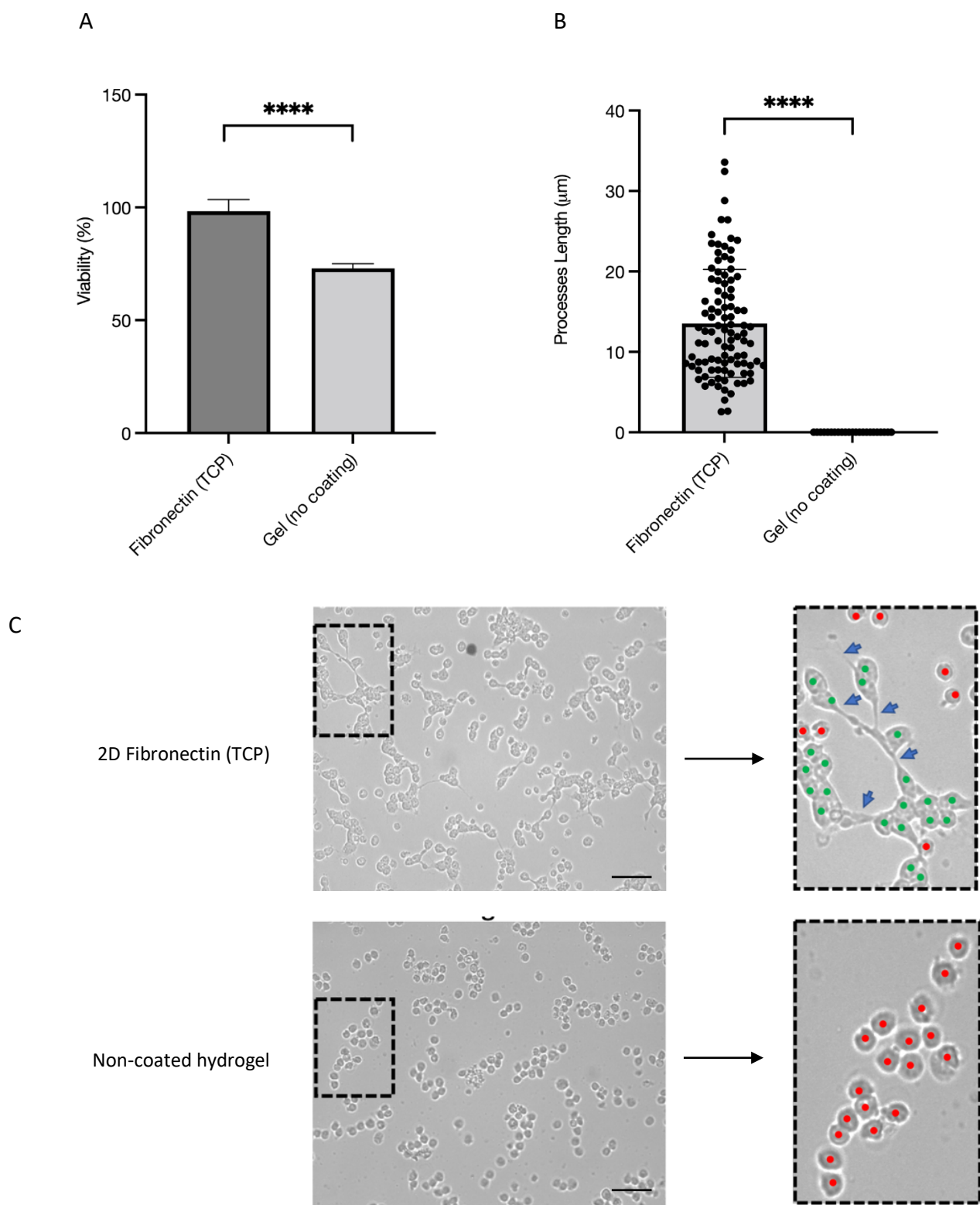


Figure 5.3: Viability and morphology of photoreceptor cells seeded on HAMA. Photoreceptor viability (A), processes length measurements (B), and microscopy (C) results of the coating comparative study between the fibronectin control (TCP) and the non-coated hydrogels. Significance was measured using an unpaired t test for (A) and (B) ($N = 1$, $n > 20$, **** $p < 0.0001$). The experiment and data analysis were performed by Dr. Aikaterini Kalargyrou. Scale bar 50 μm .

Looking at the results in Figure 5.3 (A), the viability percentages of the fibronectin control and the non-coated hydrogels were 98.3% and 73% respectively. However, viability alone is not enough to determine the success of the cell seeding on the material. Morphological signs of viable photoreceptors are also examined, including processes formation and protrusion, and are commonly studied in the literature [211], [216], [217].

This was assessed by measuring the lengths of the photoreceptor processes, as seen in Figure 5.3 (B). The cells cultures on fibronectin coated tissue culture plastic had an average process length of $13.54 \mu\text{m} \pm 6.7 \mu\text{m}$, while none of the photoreceptors cultured on the hydrogels had any processes (for the fibronectin TCP, 321 cells were images and there were 238 processes, while 105 cells where imaged on the hydrogels with no processes visible). Additionally, looking at the cell images in Figure 5.3 (C), there was a noticeable difference in the morphology of the cells. The colored dots represent healthy (green), and unhealthy (red) cells based on morphology as discussed above, while the arrows point to the processes. Again, there were no visible processes growing out of the cells cultured on the hydrogels.

It was concluded from this experiment that a coating was required on the hydrogels to drive the growth of processes. Therefore, a second experiment was conducted to test both fibronectin and laminin coatings on the hydrogels, the former having been testing extensively by the Pearson group [211], and the latter having been used in the literature for photoreceptor culture on biomaterials [98]

The second experiment examined the viability of photoreceptor cells cultured on tissue cultures plastic and on the hydrogels with the following conditions: coated with fibronectin, coated with laminin, and with no coating. Additionally, a 2D negative control was added to compare all cell viabilities to dead cell control (fixed with PFA). The conditions were tested in triplicate, and the viability was determined from the absorbance measurements of the MTS assay after the cells were cultured for 48 hours and can be seen in Figure 5.4 (A). This data was collected from three experiments.

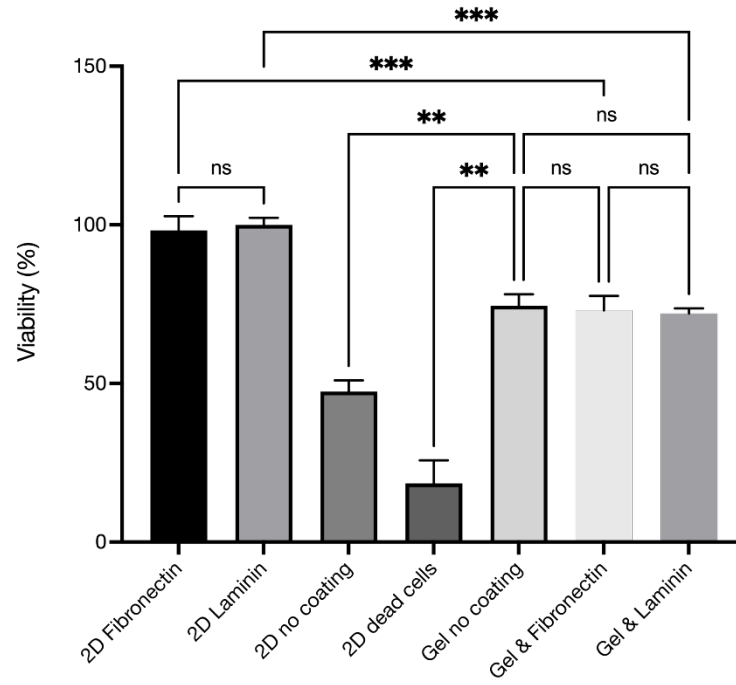
Looking at the graph, a few observations can be made. Firstly, comparing the coatings to each other, there was no significant differences in the viability of the cells cultured on the two coatings for the tissue culture plastic or for the hydrogels. This implies that both coatings were adequate for the purpose of culturing photoreceptors. To remain consistent, fibronectin was used for the remainder of the experiments, as it has been proven by the collaborators in the Pearson group to be successful at increasing the adhesion and viability of plated primary photoreceptor [211], as well as encouraging the formation of processes in photoreceptors [211], and other cell types [218].

There was a significant difference in terms of cell viability when comparing the gels with no coating to the 2D tissue culture plastic with no coating, and to the negative control. This meant that even without a coating, the cells survived better on the gel than on the non-coated tissue culture plastic.

Unexpectedly, there was no significant difference between the coated and non-coated hydrogels, with all groups exhibiting a viability of 70 to 75%. This may indicate that incubating the hydrogels with the PDL and fibronectin might not be sufficient to coat the hydrogels, and that different coating methods should be pursued to covalently bind the adhesion molecules to the surface of the hydrogel.

Finally, the cells cultured on the coated tissue cultured plastic had significantly higher viability for both types of coating, with a viability higher than 95% on the coated tissue culture plastic. Additionally, looking at the cell images in Figure 5.4 (B), there was a noticeable difference in the morphology of the cells, with no visible processes growing out of the cells cultured on the hydrogels.

A



B

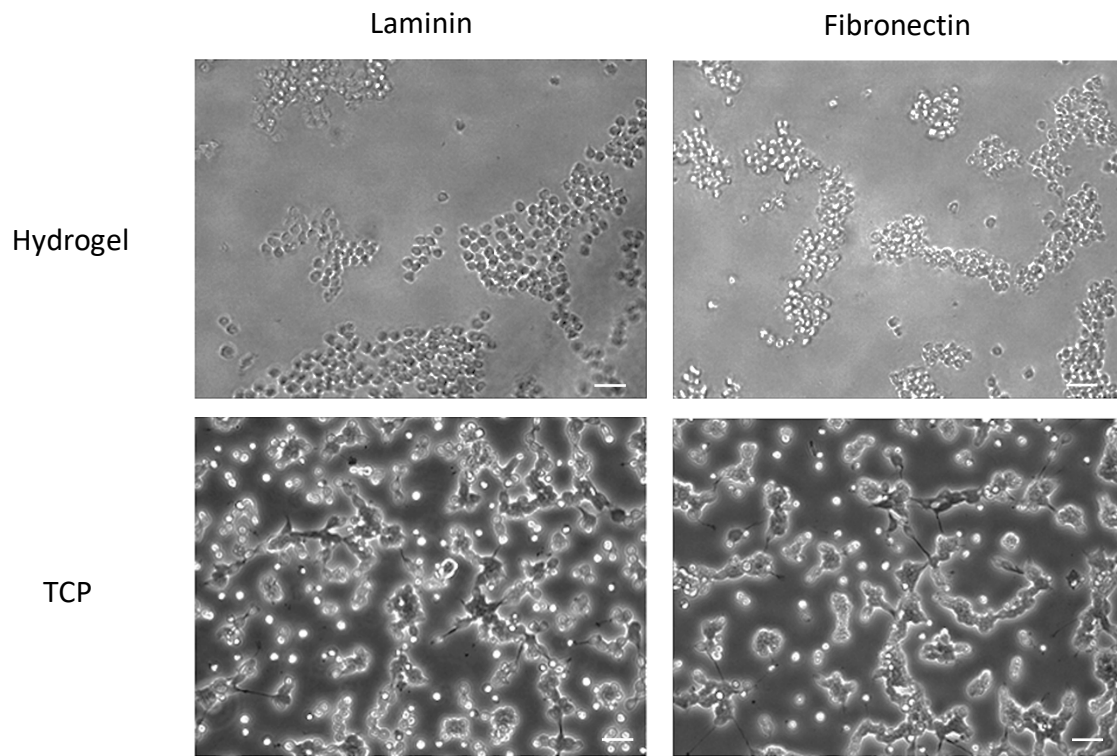


Figure 5.4: Viability and morphology of photoreceptor cells seeded on HAMA. Photoreceptor viability (A) and microscopy (B) results of the coating study between laminin and fibronectin. The experiment and data analysis were performed by Dr. Aikaterini Kalargyrou (N = 1, n > 8, ***p = 0.0002 (fibronectin), ***p = 0.0007 (laminin), **p = 0.0053 (2D no coating), **p = 0.0014 (2D dead cells)). Significance was measured using Kruskal-Wallis. Scale bar 10 μ m.

5.2.2.2.2 Testing the various HAMA – PEGDMA concentrations

The results of the coating experiments revealed that photoreceptor cells cultured on the 9% HAMA – 4% PEGDMA hydrogels displayed a high level of viability, despite being lower than those cultured on the coated tissue culture plastic. The next step was to examine the effect of hydrogel concentrations on cell viability and morphology. The initial experiment was run on 8 concentrations, in addition to the previously tested 9% HAMA – 4% PEGDMA, as seen in Table 5.1. The examined concentrations were the same as the ones studied in the swelling and rheology experiments in section 3.2, where both the PEGDMA and HAMA concentrations were screened one at a time.

Table 5.1: Possible combinations of the HAMA and PEGDMA concentrations. Examined concentrations are highlighted in blue.

		HAMA				
		3%	6%	9%	12%	15%
PEG	0%	3% - 0%	6% - 0%	9% - 0%	12% - 0%	15% - 0%
	2%	3% - 2%	6% - 2%	9% - 2%	12% - 2%	15% - 2%
	4%	3% - 4%	6% - 4%	9% - 4%	12% - 4%	15% - 4%
	8%	3% - 8%	6% - 8%	9% - 8%	12% - 8%	15% - 8%
	12%	3% - 12%	6% - 12%	9% - 12%	12% - 12%	15% - 12%

The experiment was run in a single plate, in triplicate. However, the resulting viability data was plotted in 2 graphs for ease of visualisation, as can be seen in Figure 5.5. For both the fixed PEGDMA and HAMA concentrations, there was no clear relationship between increasing the concentration of the polymer and photoreceptor viability. There were no significant differences between any of the gel groups and the control (only significant differences are between 6 – 4 and 9 – 4, and 9 – 4 and 9 – 12). Unlike the

previous test, the viability of cells on the 9% HAMA – 4% PEGDMA group was comparable to the control.

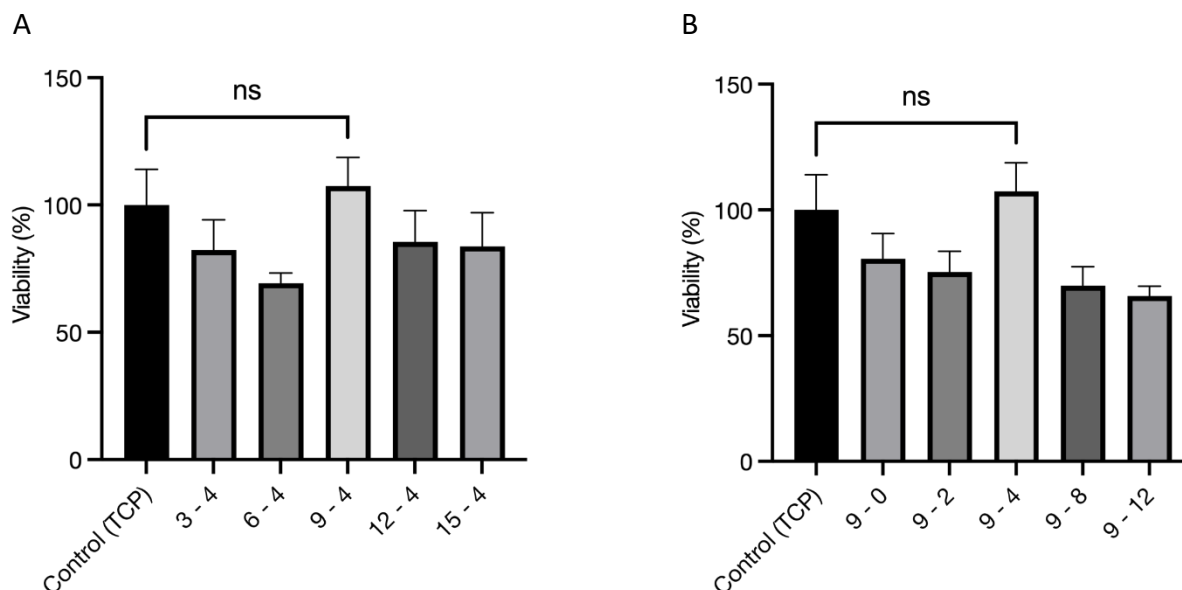


Figure 5.5: Viability of photoreceptors seeded on HAMA-PEGDMA. Hydrogels were tested with fixed PEGDMA (A) and HAMA (B) concentrations. The experiment and data analysis were performed by Dr. Aikaterini Kalargyrou (N = 1, n = 4, ns: $p > 0.05$). Significance was measured using Kruskal-Wallis.

The final experiment was run on all 25 gel combinations in Table 5.1, all in triplicate. An additional control was conducted by our collaborators as they had concerns about the Ibidi plate used. Specifically, the geometry of the wells of the angiogenesis plate presents a different volume of media to the cells compared to regular 96-well plates which may affect oxygenation [219]. Therefore, they performed a separate tissue culture control on a regular 96 well plate to see if there was any difference between the two.

The viability percentages of the photoreceptors on the different hydrogel concentrations and the controls, as well as low magnification microscopy images of the cells, can be seen in Figure 5.6. Interestingly, the only significant difference in viability was between

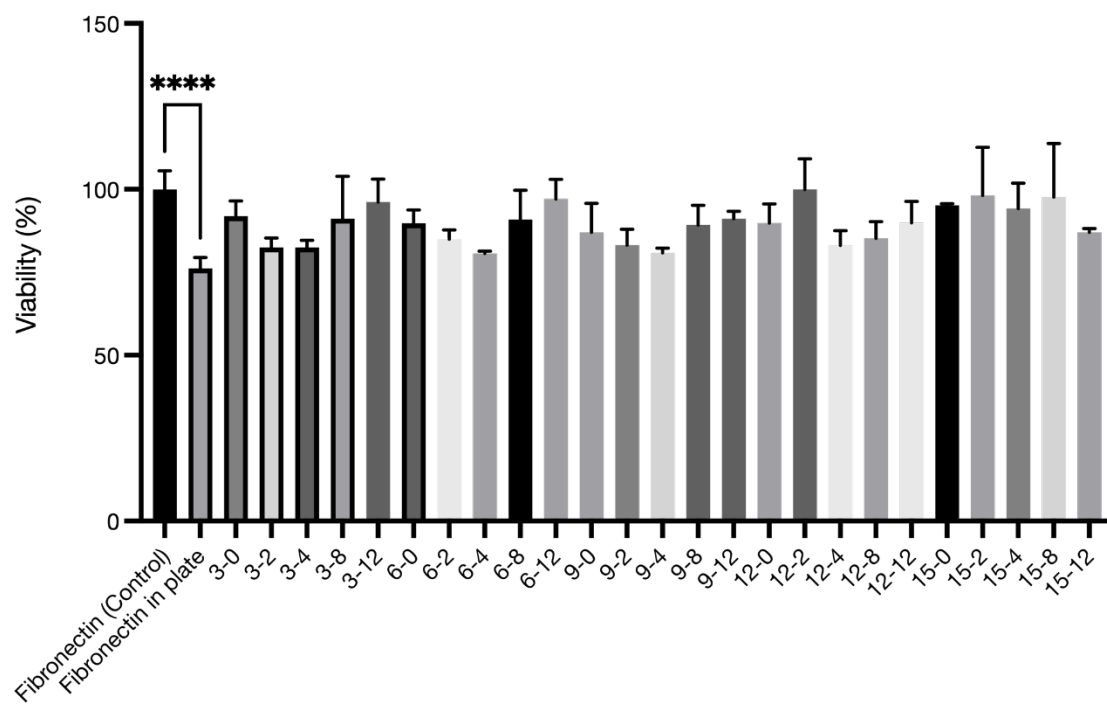
the 2 controls. The internal tissue culture plastic control on the Ibidi plate appeared to have a significantly lower cell viability percentage compared to the external control on a regular 96 well plate. Therefore, the plate choice used for these experiments should be reconsidered in future experiments.

The microscopy images reveal the hydrogel conditions (6 – 4, 6 – 8, 9 – 2, 9 – 4, 9 – 8) where the cells exhibit the healthiest distribution (equally distributed over the surface of the gel). Again, the combination 9% HAMA – 4% PEGDMA exhibited promising results, which aligned with the results of Chapter 3, making it the preferred choice to move forward with.

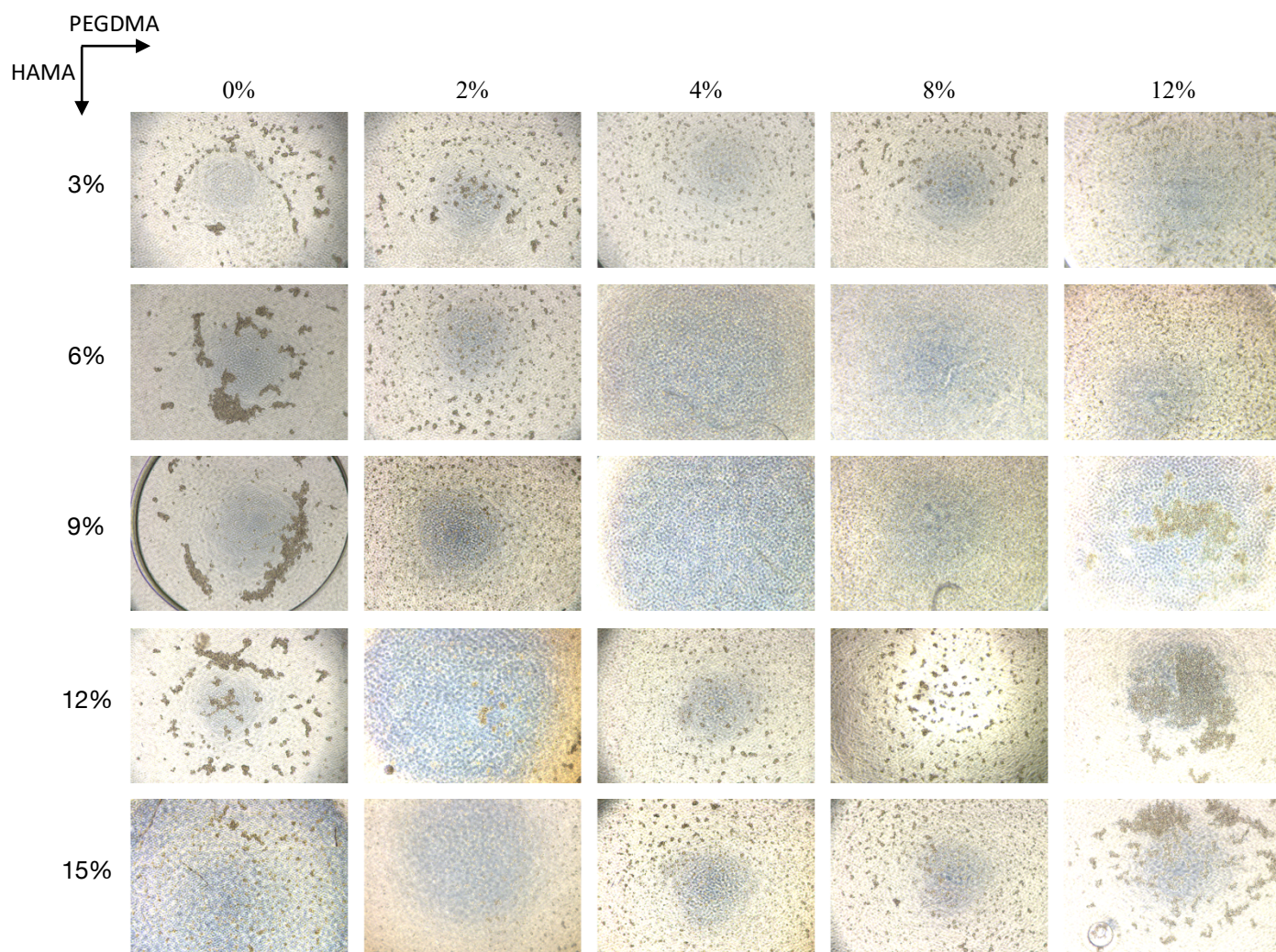
This experiment was repeated; however, the second iteration had a large amount of bacterial contamination in the plate, making the results invalid. Further optimisation is required to ensure that the hydrogel is sterile and therefore avoid any further contaminations.

Figure 5.6: Viability and morphology of photoreceptors seeded on HAMA-PEGDMA. Photoreceptor viability (A) and microscopy (B) results for all the combinations of concentrations. The experiment, microscopy, and data analysis were performed by Dr. Aikaterini Kalargyrou (N = 1, n > 3, ****p < 0.0001). Significance was measured using Kruskal-Wallis. Scale bar 10 μ m. The figure can be seen on the next page.

A



B



5.3 Photoreceptor cultures with pre-incubated media

Following the results of the cell culture experiments on flat hydrogels, further cytotoxicity experiments were conducted. Using a modified protocol from Masters *et al.* (2005) [141], photoreceptors were cultured on coated tissue culture plastic, and incubated with media that was pre-incubated with hydrogels with or without hyaluronidase enzyme. These tests were only conducted with the 9% HAMA - 4% PEGDMA condition. This experiment was performed to determine if the degradation products, or any material that might have passively leached out of the hydrogels, affected the cell viability and morphology.

5.3.1 Materials and methods

5.3.1.1 Preparing the pre-incubated solutions

The gel precursor solution was prepared by weighing lyophilized HAMA, PEGDMA and LAP into separate Eppendorf tubes. Sterile 1X PBS was added to the LAP, and that solution was added to the PEG and vortexed until the PEG was fully dissolved. Following that, the new solution mixture was pipetted on top of the HAMA and left overnight at 4°C to dissolve the lyophilised HAMA. The tubes were kept covered in aluminium foil to prevent any polymerisation from ambient light.

The following day, 40 µL of the HAMA-PEG precursor solution was pipetted into sterile 1 mL syringes, with the top of the syringe already cut off, and crosslinked for 15 minutes under a 365 nm UV lamp. The gels were then placed in a 48 well plate, washed 3 times with PBS, and left for an hour under the UV lamp to sterilise the hydrogels. Finally, the gels were washed 3 times with photoreceptor media to remove any unbound gel materials still adhered to the surface of the gel. The plate was left overnight in the incubator in photoreceptor media to facilitate solvent exchange withing the gels.

Several incubation conditions were tested. The hydrogels were incubated with either a solution of 22 unit/mL of hyaluronidase in photoreceptor media, matching the value used in the degradation experiment, or with photoreceptor media. Additionally, controls of

both the buffer and the enzyme solutions were included. All four of these conditions were incubated for 24, 48 and 72 hours, following which the media was collected. All hydrogel conditions were run in triplicates.

5.3.1.2 Photoreceptor culture protocol

The photoreceptors were dissociated according to the protocol described in section 5.1. The cells were seeded in the 96 well plate at a concentration of 2 million cells/mL and incubated with fresh photoreceptor media for 24 hours at 37 °C and 5% CO₂. This was to ensure that the cells were adhered to the plate before adding the pre-incubated solutions. The following day, the cell culture media was removed and replaced with the pre-incubated solutions, and the plate was incubated for 48 hours. Cell experiments were conducted in triplicate.

Once the incubation period had passed, 20 µL of the cell proliferation assay MTS reagent was added to each well, and the plate was incubated for 4 hours at 37 °C and 5% CO₂. The plate was placed on a shaker for 1 minute, following which the absorbance of the samples was measured on a plate reader at 490 nm against a blank control. This assay was performed to determine the viability of the cells.

Once the assay was completed and the measurements taken, the cells were fixed with 4% PFA and stained with Trypan Blue to visualise the cell morphology and check for processes formation. Microscopy images of all the wells were taken, however only one representative image per condition is shown in Figure 5.8. Since the cells were fixed, trypan blue stained the entire cell, as the fixation process permeabilised their membrane. More importantly, trypan blue stained the processes with a strong enough contrast to be observed under a standard brightfield microscope, which was the purpose of this step of the experiment.

5.3.2 Results and discussion

The viability of the photoreceptors was plotted for the different conditions and time points, and the experiment was repeated 3 times, as seen in Figure 5.7. The viability was determined from the absorbance measurements of the MTS assay.

After running a Kruskal-Wallis test on all three experiment, the majority of the conditions (except those indicated in the plots) did not yield significantly different results when compared to the corresponding tissue culture plastic control. Therefore, the pre-incubated solutions did not affect the viability of the photoreceptor cells cultured.

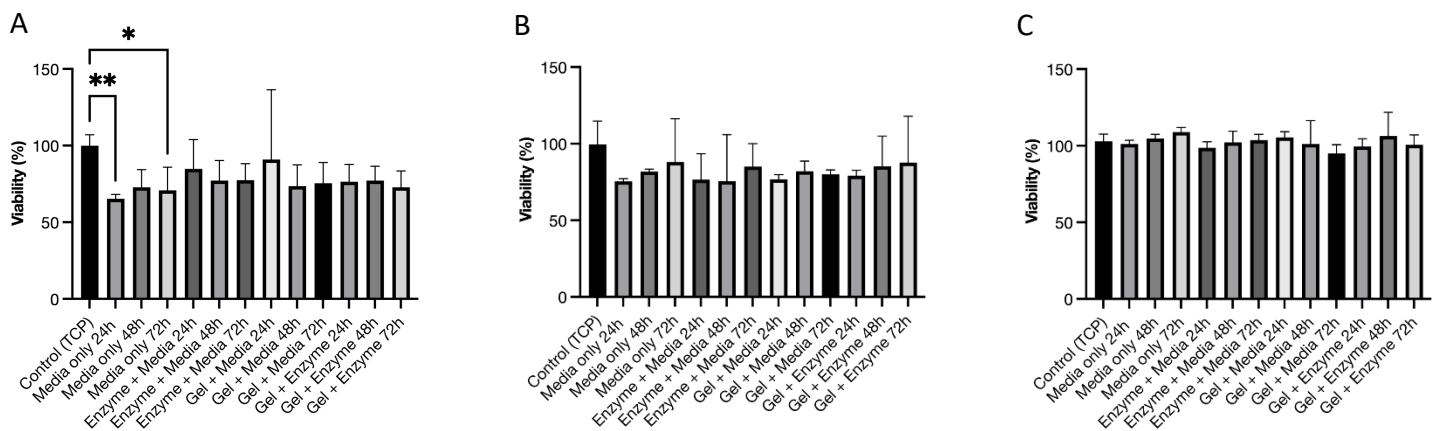


Figure 5.7: Photoreceptor viability results for the three experimental repeats. The experiment and data analysis were performed by Dr. Aikaterini Kalargyrou (N = 3, n > 8, **p = 0.0077, *p = 0.0408) . Significance was measured using Kruskal-Wallis.

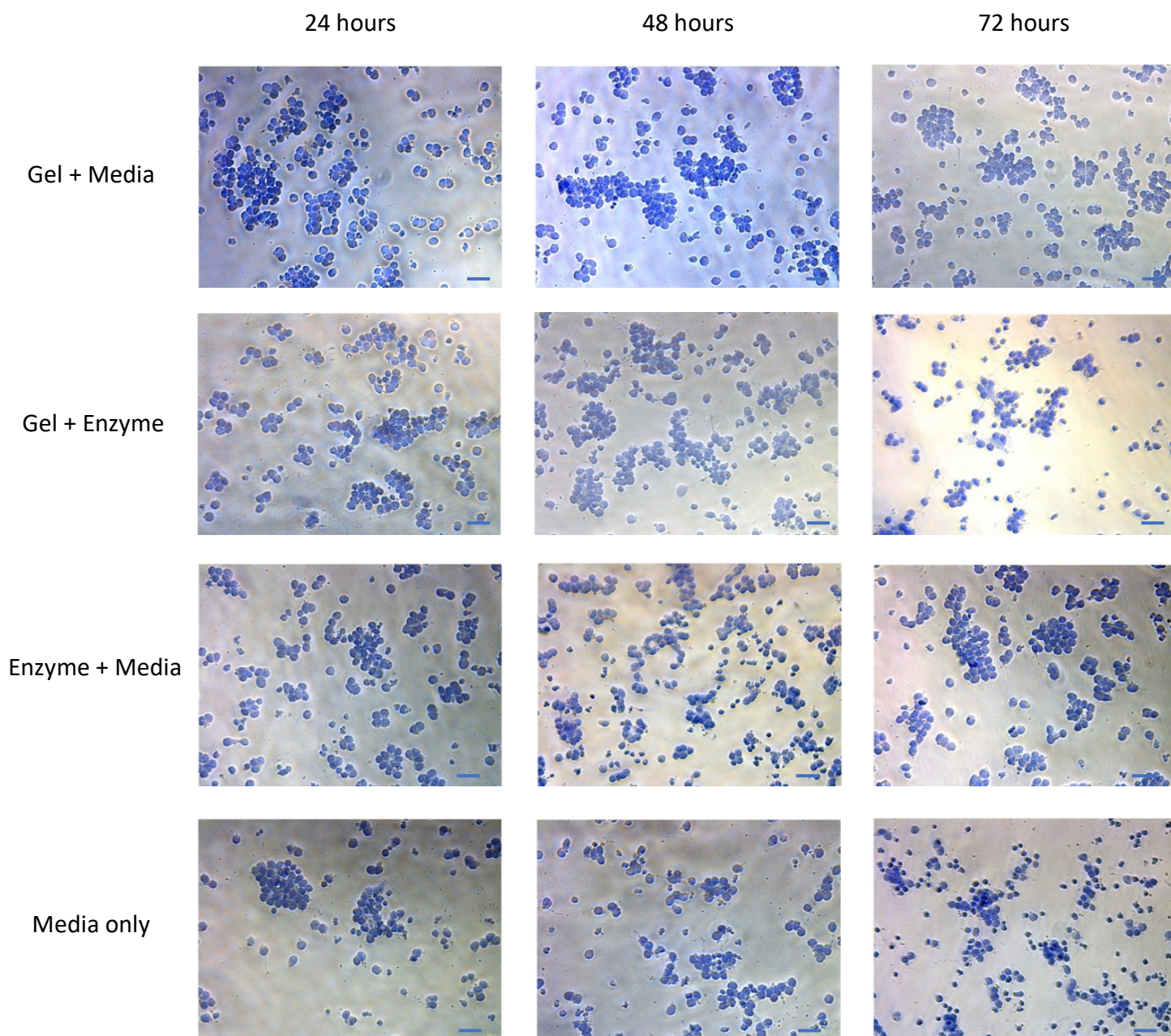


Figure 5.8: Photoreceptor microscopy results for all the combinations of concentrations. The experiment and microscopy were performed by Dr. Aikaterini Kalargyrou. Scale bar 10 μm .

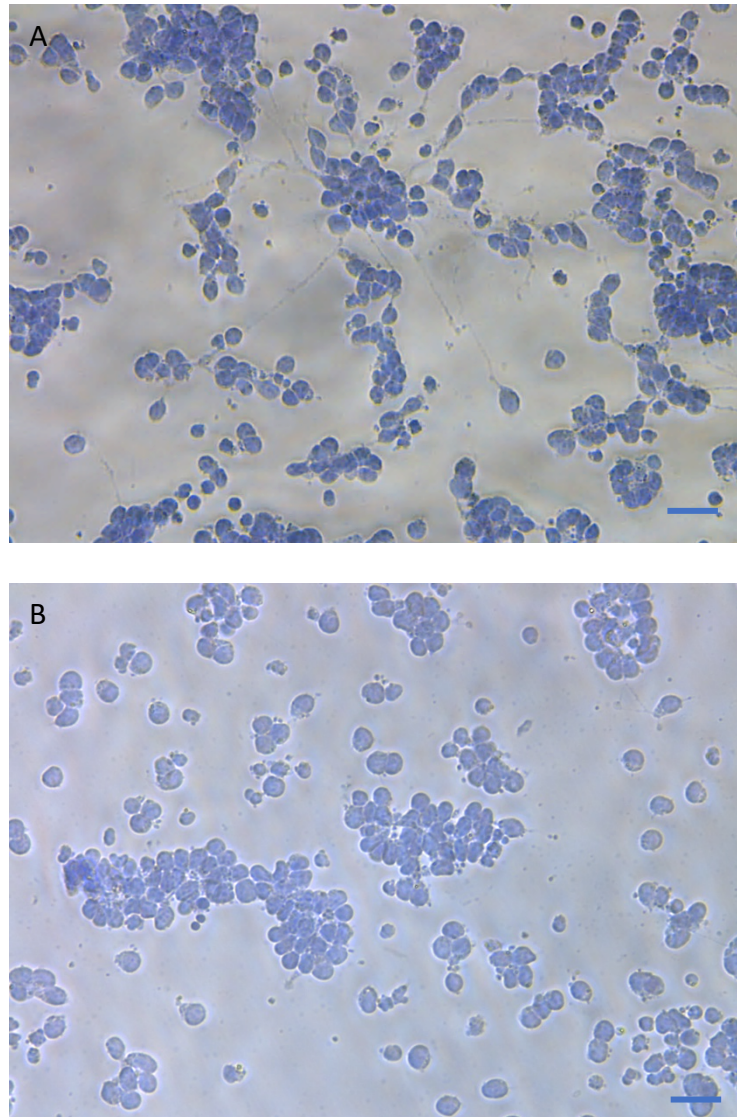


Figure 5.9: Photoreceptor microscopy results compared to control. The fresh media control (A) was compared to the 48 hours gel in media condition (B). The experiment and microscopy were performed by Dr. Aikaterini Kalargyrou. Scale bar 10 μm .

However, looking at the images in Figure 5.8, most of the cells were round, and there was a small amount to processes growing. When fresh media control is compared to the

hydrogel conditions, it is clear that the cells were not as healthy and there was significantly less processes growing with the pre-incubated media.

Technically, the pre-incubated media with hydrogels should be compared to the internal pre-incubated media only controls, instead of an external fresh media control, however those two pre-incubated conditions look very similar. Also, the internal control had much less processes when compared to the external control, even though they were both just photoreceptor media, with the only difference being that one was incubated for a certain amount of time.

5.4 Conclusion

The results of the cell culture experiments on flat gels demonstrated that the cells could remain viable for up to 48 hours after being seeding on to both GelMA and HAMA hydrogels, crosslinked with and without PEGDMA, at different concentrations. More in-depth cell morphological analysis of cells cultured on the HAMA – PEGDMA hydrogels revealed that the cell morphology was not ideal, and that none of the cells appeared to grow any processes.

Therefore, to understand whether the lack of cell processes was due to the presence of cytotoxic products released from the hydrogels, the cells were cultured on tissue culture plastic, and incubate with pre-incubated media. Unfortunately, the results of this experiment did not yield a clear conclusion as to why the cells were not as healthy on the hydrogels when compared to the tissue culture plastic controls. The initial analysis suggested that any material the hydrogels released into the media, as well as the hydrogel degradation products, were not toxic to the photoreceptors. However, the representative microscopy images showed limited processes growing out of the cells with the pre-incubated media conditions. Knowing that these cells were seeded for 24 hours before the tested media was added, it could be possible that any processes that had grown in those 24 hours were affected as well. More tests need to be conducted to

better understand this photoreceptor behaviour, particularly since process growth is a crucial aspect of this scaffold design.

Chapter 6: Conclusion and future work

Irreversible loss of photoreceptors, due to age-related macular degeneration (dry and wet) or inherited retinal diseases, is one of the main causes for retinal degenerative diseases. This results in visual impairment that progresses over the years and can even cause blindness. Several different treatments to treat macular degeneration diseases have been studied to halt the progression of the diseases, with injections of VEGF inhibitors being a commonly used and approved therapy [7], [8], and some gene therapy treatments showing great potential [24], [25]. However, there are limited clinical treatments for restoring normal vision in patients affected by macular disorders, especially at late stages of the disease, and the treatments that do, such as prostheses, provide low resolution vision [35].

Therefore, alternative strategies to address these disorders and potentially restore the damaged vision have been investigated. Various cell replacement therapies for retinal degeneration have been studied over the past two decades with different degrees of success. These therapies deliver photoreceptors, sourced from either donors or pluripotent stem cells, into the diseased retina, in the form of cell suspension injections or retinal sheet transplantations [7].

Early publications reported evidence of photoreceptor survival and integration in the host retina [39], [40], however many failed to differentiate into mature photoreceptors [42], [44], [77]. Papers such as MacLaren *et al.* (2006) and Akimoto *et al.* (2006) paved the way for more successful transplantations [45], [47]–[49] by identifying the optimal stage for donor photoreceptor isolation. In 2011, the Sasai group developed a ground-breaking to differentiate ECSs into retinal organoids, driving the field forward [3], [60], [62]. However, despite the promising results reported by several research groups [51], [63], [64], [68], findings by Pearson *et al.* (2016) bring into question the results previously reported [65], [73].

More recent literature has demonstrated the advantages of using biomaterial support and scaffolds compared to bolus injections. These scaffolds can facilitate the transplantation of the donor photoreceptor cells into the retina in a more controlled and structured way and have been shown to increase cell viability and integration [74], [86]. Additionally, hyaluronic acid hydrogels have been shown to distribute the transplanted cells more evenly in the host retina, and increase their viability [40], [42], [90]. Researchers have also pursued more complex scaffolds, with patterned geometries, for the purpose of aligning photoreceptor cells *in vitro* prior to transplantation, with promising results published by several groups in the last decade [98], [102]. Most notably, Jung *et al.* (2018) established a unique scaffold design, that showed promising photoreceptor alignment, and the presence of pre-synaptic markers at the bottom of their channels. This suggested that the photoreceptor cells were primed for synaptic formation, which had not been shown in any of the other publications thus far [98]. These results have influenced the studies described in the rest of this thesis.

The first scaffold geometry fabricated was the ‘wine glass’ channel design, inspired by Jung *et al.* (2018) [98]. Using photolithography and a dry etching process, a master mould was successfully produced, with various channel diameters and spacings. The PDMS negative was then fabricated using soft lithography, followed by a demoulded patterned GelMA scaffold. Although the initial scaffold fabrication attempts were successful, this channel design had a significant limitation. Theoretically, each channel should be able to hold approximately 8 cells, however practically, Jung *et al.* were only able to fit 2 to 3 cells per channel on average [98]. With these photoreceptor packing numbers, these scaffolds could not replicate closely enough the density of the photoreceptor layer in the native retina, which is about 8 to 10 cells deep [173].

Therefore, the scaffold geometry was modified to the ‘champagne flute’ channel design. This adjustment would allow the scaffold to hold a higher density of photoreceptor cells per channel, theoretically up to 30 cells per channel, increasing the total number of photoreceptors in the scaffold. The negative moulds were successfully fabricated using

a high resolution two photon polymerisation printer, resulting in a highly defined negative print with columnar structures. The printing process required some optimisation, including tuning the printing parameters and the cleaning process of the mould, as well as determining the best 3D design features of the mould such as spacing, total print height and array geometry.

Additionally, some preliminary hydrogel scaffolds were also demoulded. Although GelMA did show promising results, a choice was made to change the biomaterial from GelMA to HAMA, motivated by several publications highlighting the positive role HAMA hydrogels have played in increasing photoreceptor viability [42], [90], [92]. At this stage, the scaffold still required more optimisation, however, some material characterisation needed to be performed before proceeding.

The hydrogels were characterized to determine the optimal polymer concentrations for the desired physical properties of the scaffold, with four different studies: swelling, rheology, diffusion, and degradation.

The swelling behaviour of the hydrogel scaffold was examined as it is vital that the swelling of the patterned gel is minimised to preserve the patterned geometry. Experiments indicated that it was possible to tune the concentrations of the hydrogel to produce a minimally swelling scaffold, with several of the polymer formulations. Swelling experiments are important in the literature and have been performed on pure HAMA and HAMA – PEG hydrogels, reporting similar results to the ones obtained, confirming that swelling can be decreased by increasing polymer concentrations, and with addition of a co-polymer such as PEG [124], [141]. Another approach to measure swelling in a patterned hydrogel was described in Shrestha *et al.* (2020). In this paper, the authors determined the volume swelling ratio, instead of the more commonly measured mass swelling ratio, by imaging the scaffold in 1 μm slices, measuring the area of each slice, and calculating the total volume of the scaffold [102]. This approach could be interesting to use in future experiments, as it provides more information regarding the final volume

of the hydrogel compared to the theoretical scaffold volume, as well as the final geometry of the pattern and diameters of the channels.

Following this, rheology experiments were conducted on the hydrogels to measure the storage and loss moduli of the various conditions. The PEGDMA and the HAMA screening experiments both showed that with increasing concentrations of polymers the storage modulus increases. This was attributed to the higher crosslinking density of polymers, which in turn increases the storage modulus of the gels [124]. These results also showed that it was possible to fabricate a wide range of hydrogels with tuneable mechanical properties.

Pilot diffusion experiments were also performed to verify that the material was sufficiently porous to allow for small particles to diffuse, with two molecular weight dextran molecules tested as representative molecules. It was determined that the gels with the lowest polymer concentrations had the fastest diffusion rates. This was attributed to the low crosslinking density and therefore the large pores within the hydrogel structure [124], [141]. Further studies with a wider range of dextran molecules should be performed to determine the limit of diffusion. More importantly, this experiment was not representative of the *in vivo* scenario, in which the molecules would diffuse across the entire thickness of the hydrogel, instead of outwards from within the hydrogel. To replicate more closely the *in vivo* diffusion across the patterned hydrogel, experiments with a diffusion vessel, like the one described in Mignot 1990 [197], and performed in several papers described [112], [196], [198], should be conducted in the future.

The final hydrogel characterisation experiments were performed to study the degradation behaviour of the various hydrogel concentrations when incubated with hyaluronidase. The hydrogels were incubated in a solution of 22 units/mL of hyaluronidase, matching the measured concentration of this enzyme in the human vitreous [208]. The solution was replaced daily to maintain the enzyme activity over the course of the experiment. The degradation was quantified using a Micro-BCA™ Protein

Assay Kit to measure the amount of protein released in the solution [112] This experiment demonstrated that it was possible to fabricate hydrogels with tuneable degradation rates, whereby lower concentrations of polymer had faster degradation rates. As the assay was incapable of detecting all the observed degradation, further studies should be performed to understand the reason behind it. Additionally, hyaluronidase concentrations may vary in animal and human models, therefore some *in vivo* experiments should also be conducted.

The hydrogel characterisation experiments performed here indicate that the 9% HAMA, 4% PEGDMA and 1% LAP formulation was the most favourable for use in patterned scaffold fabrications. However, several of the tested formulations could also be used, providing a range of degradation rates and hydrogel stiffnesses. Furthermore, these experiments were conducted on a non-patterned hydrogel and should be repeated in the future on the final patterned scaffold to determine how the pattern affects these properties and therefore the final formulation selection.

Once the optimal hydrogel concentration was determined, further optimisation of the scaffold fabrication process was performed to successfully and repeatably produce the final scaffold in a semi-automated manner. The first experiments focused on producing a scaffold with the desired pattern, and more specifically, with the designed channel geometry and no excess hydrogel material. These required increasing the height of the negative mould to 215 μm , 15 μm thicker than the spacer used. This was unexpected and could not be determined intuitively, but instead required trial and error to identify this optimal print height. Following this, the demoulding process was optimised, and the setup was adapted to a uniaxial loading machine to perform a force-controlled demoulding. All the fabricated scaffolds were produced from a solution of 9% HAMA, 4% PEGDMA, and 1% LAP. Further testing should be performed on the other hydrogel concentrations to determine if the same demoulding parameters can be used, or if each hydrogel concentration requires optimisation of the fabrication process.

In the final experimental chapter of this thesis, preliminary cell viability experiments were conducted to examine the effect of the hydrogel formulation on cell survival *in vitro*. These experiments were conducted on flat gels and demonstrated that the cells could remain viable for up to 48 hours after being seeded on the hydrogel, including various concentrations of GelMA and HAMA, crosslinked with and without PEGDMA. However, more in-depth morphological analysis of cells cultured on the HAMA – PEGDMA hydrogels revealed that the photoreceptor cell morphology was not ideal, as all the cells were round, and none appeared to grow or extend any processes.

Further experiments were performed to understand whether the observed lack of processes was due to cytotoxic products released from the hydrogels, which could include degradation products or material that may have passively leached out. Photoreceptor cells were cultured on coated tissue culture plastic and incubated with media that was pre-incubated with hydrogels with or without hyaluronidase enzyme [141]. Unfortunately, the results of these experiments did not yield a clear conclusion as to why most of the cells had a round morphology on the hydrogels when compared to the tissue culture plastic, and why the cells had limited processes growing out. Further tests need to be performed to better understand this behaviour. Additionally, alternative cell culture plates should be considered, as it was suggested that the Ibidi ones chosen could be inhibiting oxygenation of the cultured cells.

In the literature, most papers that used primary mouse photoreceptors isolated the cells and directly transplanted them into the animal model. It wasn't until Mitrousis *et al.* (2016) that a study of these cells cultured on materials was published [90]. In general, the literature available on primary photoreceptor culture *in vitro* is limited [220], [221].

The goal of this thesis was to produce hydrogels with micropatterned channels designed to polarise photoreceptors. Therefore, further experiments are required to determine if it is possible to control the polarisation of the cultured photoreceptors. Furthermore, a co-culture setup should be developed to culture both photoreceptor and RPE cells together.

This has been shown to increase the viability of both cell types, and when transplanted together, there was evidence of visual recovery [92].

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