A Microfabricated 3-D Stem Cell Delivery Scaffold
for Retinal Regenerative Therapy

by

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ABSTRACT

Diseases affecting the retina, such as Age-related Macular Degeneration (AMD) and Retinitis Pigmentosa (RP), result in the degeneration of the photoreceptor cells and can ultimately lead to blindness in patients. There is currently no cure for AMD or RP, and only a few methods exist for slowing the progression of these diseases. Although there has been much recent headway in cell replacement therapy to restore vision loss, a number of challenges still remain. More specifically, there is a need for the development of a device that can deliver a large number of cells to the posterior segment of the eye, while promoting cell survival, differentiation and integration into the retina following transplantation. This research focuses on designing a device to meet these demands and improve the vision of those afflicted with blinding diseases.

The specific hypothesis behind the proposed research is that a MEMS-based strategy to engineer a device can provide precisely defined spatial and chemical cues to influence retinal progenitor cells (RPCs) attachment, promote differentiation, and provide physical guidance in a more normal anatomical organization for their integration as neurosensory retina after transplantation to the subretinal space. Therefore, the specific aims of this research are to design, fabricate, and evaluate in vitro a novel ultrathin 3-D device made of polycaprolactone (PCL) for retinal cell replacement synthesized by the stacking, aligning, and bonding of three uniquely designed layers. Photolithography, standard replica molding, and soft lithography techniques are used to fabricate the device elements.

The 3-D device is designed with a defined cage structure to encapsulate a large number of cells. Another layer of the design allows for unidirectional cell migration out of one end into the subretinal space with the aid of contact guidance ridges. The third design layer allows for nutrient infiltration from the retinal pigment epithelium into the cell cages. The ultimate goal is to provide an environment compatible with the normal retinal tissue and conducive to the formation of functional synapses under the appropriate conditions, thereby restoring proper vision. With demonstration of efficacy and cell retention in vitro, the scaffold has the potential to reverse retinal degeneration due to disease or trauma and improve retinal function and integrity in vivo.

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ASSIGNMENT

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Chapter 1: Introduction

1.1 An Introduction to Regenerative Therapy

The solution to some of the most pressing medical challenges may lie in the promise of regenerative medicine. New strategies to regenerate tissues and organs for the treatment of injuries and diseases have the potential to resolve the worldwide problem of organ shortages. Though many elements of the human body such as skin and blood are naturally regenerative, diseases and serious injuries can be so damaging that they result in complete loss of functionality without the potential for regeneration. Recent figures claim that an estimated 97,000 people in the United States awaited life-saving organ transplants in 2007\(^1\). Given that there were approximately 28,000 transplants total performed in the United States that year\(^2\), it is clear that the demand for organs cannot be met at the current donation rate. Of all those who do not receive organs, 17 of them die each day while waiting. Furthermore, the wait for an organ is three to five years on average, as each day the wait list grows five times faster than the donation list. \(^1\)

To meet the demand for tissues and organs in an efficient and safe manner, strategies such as regenerative medicine may be the solution. Regenerative therapy is a multidisciplinary effort- it requires integration of concepts from biology, chemistry, materials science, engineering, and medicine, among others. The underlying strategy is consistent throughout all applications of the therapy. Cells from a biopsy are used to form a monolayer cell culture and subsequently an expanded line of cells. These cells are then cultured on a 2D or 3-D polymeric scaffold for the generation of a graft. The graft is then transplanted to the relevant site in the host tissue or organ to induce regeneration. With the appropriate host environment and therapeutic signals, the tissue regenerates to restore function and is nearly indistinguishable from native tissue.

In theory, the optimized combination of biomaterials and polymers, stem cell or progenitor cell populations, and growth factors will induce generation of tissues and organs. There has been much recent headway in the engineering of skin,
cartilage, liver, bone, and heart valves, among other tissues. Though regenerative medicine is fairly straightforward in principle, it can be very challenging to implement in practice. Optimization of the scaffold materials, cells, and therapeutic agents require extensive *in vitro* and *in vivo* testing before the possibility of transplantation into humans.

### 1.2 Biology of the Retina

The retina is the multi-layered sensory tissue that lines the back of the eye, as shown in Figure 1-1. The thickness of the retina ranges from .1 mm to .56 mm depending on the region.³ The retina is composed of approximately seven distinct layers, as shown in the figure. The photoreceptors in the retina capture light rays and convert them to electrical impulses that travel along the optic nerve. The impulses are then converted to images in the brain. The photoreceptors, namely the rods and cones, are central to the maintenance of healthy vision. There are approximately 6 million cones in the macula, or the center of the retina responsible for central vision. The cones allow for detection of color. The retina contains 125 million rods, which are responsible for night vision, spread throughout the periphery of the retina.⁴

![FIGURE 1-1: Schematic of the eye and the retina. ⁵, ⁶](image)

*Source of eye diagram: American Academy of Ophthalmology*
In designing a device for stem cell delivery to the posterior segment of the retina, consideration of the retinal microenvironment is essential. The scaffold must be very thin, given that the retina itself is only 100 to 560 μm thick. A thinner scaffold will be less disruptive to the host tissue anatomy and less invasive on the surrounding structures. The subretinal space is artificially created during the transplant procedure. Therefore, thick implants greater than 30 μm will induce trauma during transplantation and ultimately interfere with the normal functioning of other layers of the retina. In addition, it is important to appreciate that the retina is a polarized tissue that lies on the retinal pigment epithelium on one end. The other end of the retina is the optic nerve fiber layer that absorbs the incident light. Furthermore, in order to maintain the mechanical uniformity and stability of the retina during the regenerative process, the scaffold should exhibit mechanical properties comparable to that of the retina. For example, the retina’s elastic modulus is 0.1 MPa and maximum strain at failure is 83%. However, the scaffold ultimately degrades, and so the retinal regenerative therapy does not hinge on the mechanical properties of the device.

1.3 Retinal Diseases

Retinal diseases vary widely in terms of frequency, symptoms, diagnosis, and treatment. Some of the most common retinal diseases include Retinal Detachment, Retinal Tears, Diabetic Retinopathy, Retinal Vascular Occlusion, and Macular Holes, among others. Two retinal diseases that ultimately lead to blindness and for which current treatment options are limited are Age-related Macular Degeneration (AMD), the most common cause of legal blindness among older Americans, and Retinitis Pigmentosa (RP), a rarer, inherited disease. These diseases are caused by degeneration of the retina over time. For this reason, they are appropriate targets for strategies of retinal regenerative therapy.
1.3.1 Age-Related Macular Degeneration

AMD is the leading cause of blindness in Americans age 65 and older. More than 1.75 million people in the U.S. have AMD, and this number is expected to reach almost 3 million by 2020 due to the increasing size of the elderly population. The disease is the result of genetic and environmental factors, and it gradually results in the loss of sharp, central vision with age, as shown in Figure 1-2.

![Normal Vision vs Vision with AMD](image)

**FIGURE 1-2:** Deterioration of vision associated with AMD. Source: National Eye Institute, National Institutes of Health

The loss of central vision occurs due to degeneration of the macula, the center of the retina that allows one to see detail, as shown in Figure 1-3. Women are slightly more likely to develop AMD than men. A common symptom of AMD is the presence of drusen, or yellow deposits under the retina around the macular region. Though AMD does not result in pain, the effects on vision can be very detrimental to the quality of life.

![Pathology associated with AMD](image)

**FIGURE 1-3:** Pathology associated with AMD.
There are two types of AMD, dry (non-neovascular) and wet (neovascular). 90% of patients with AMD suffer from the dry form of the disease, which involves the slow and gradual shrinkage of the retina and degeneration of retinal layers. The patients suffer severe visual handicaps due to the gradual loss of central vision. The remaining 10% of patients with AMD have the wet form of the disease. This type is the more serious form and leads to 90% of the blindness associated with the disease. In wet AMD, new blood vessels grow under the retina and leak blood and fluid. This fluid permanently damages the light-sensitive retinal cells and results in central vision loss. Factors associated with AMD are aging, obesity, heredity, hypertension, smoking, lighter eye color, and drug side effects.

There is currently no cure for AMD, and no way to completely stop its development. However, there are some treatments that may slow the progression of disease. There is no FDA-approved treatment for dry AMD, but Vitamins A, C, E, and other nutritional supplements, as well as sunglasses with UV protection, may slow its progression. Treatments for wet AMD that are aimed at stopping abnormal blood vessel growth include FDA-approved drugs Lucentis, Macugen, Avastin, and Visudyne used with Photodynamic Therapy.

1.3.2 Retinitis Pigmentosa

Retinitis pigmentosa (RP) is a rare, inherited disease that affects approximately 80,000 people in the United States, or 1 in 3700. In RP, the rods that are responsible for night vision in dim light gradually deteriorate, making night vision poor. As shown in Figure 1-4, peripheral vision also deteriorates. Only a small area of central vision, or tunnel vision, remains as the disease progresses ultimately towards blindness.

Similar to dry AMD, there are currently no treatments available for RP, although supplements of Vitamin A may delay the vision loss. Individuals with RP might also consider using low vision devices that help to magnify and illuminate objects. Clearly, there is a need for treatment of retinal diseases that deteriorate photoreceptor cells and result in blindness.
Although no visually beneficial treatments exist for AMD and RP, a number of approaches are under investigation, including the development of cell-scaffold composites for tissue engineering. Retinal pigment epithelium (RPE) transplants and retinal prosthesis are also being developed to enhance retinal function in patients suffering from retinal degenerative disease.

1.4 Current Status of Retinal Progenitor Cell Grafting

Photoreceptor loss and retinal degeneration are currently untreatable conditions. However, there are several therapies in development that employ a variety of strategies. These therapies include delivery of stem or progenitor cells to the outer retina, design of bulk scaffolds for stem cell delivery, and design of MEMS-based and nanostructured 2-D scaffolds.

1.4.1 Injection-based Cell Delivery

To successfully graft retinal progenitor cells (RPCs) to the retina requires overcoming challenges of delivery methods, cell survival, migration, and differentiation, among other obstacles. Some of the earlier approaches for retinal tissue engineering consisted of the bolus injection of a cell suspension into the subretinal space.\(^\text{17}\) However, delivering cell suspensions into the vitreous cavity or subretinal space by injection does not lead to high levels of cell survival due to the
shear forces involved in the process. Furthermore, the cells create a disorganized array and often localize to the wrong region of the retina.

### 1.4.2 Scaffold-based Cell Delivery

Subsequent research found that delivering the cells on a scaffold enhances cell survival and differentiation ability. A cornerstone of tissue engineering is the design of scaffolds that stem cells can be cultured on and the subsequent delivery of the cell-scaffold composite to the pertinent site of degeneration. In the case of retinal tissue engineering, stem cells or retinal progenitor cells (RPCs) are cultured on scaffolds and transplanted to the subretinal space. It is theorized that this process promotes cell survival, integration, and differentiation. However, the scaffold must be biocompatible and mechanically compatible with the retina in order for use in transplantation. Many recent advances in materials processing and fabrication allow for precise control of scaffold design and material parameters in order to mimic native tissue microenvironments. Regulation of properties such as topography, adhesion molecules, and soluble factors allows better control of cell behavior and host tissue response.

#### 1.4.2.1 Irregular Bulk Scaffolds

Surface modifications can be utilized to control responses at the biological interface of synthetic or natural biomaterials upon implantation. Changes in architecture and surface topography can result in a material that closely mimics native tissue responses. For example, one study evaluated degradable PLA/PLGA scaffolds for their effects on the survival and differentiation of retinal progenitor cells. The scaffolds were fabricated by phase-inversion casting and solid-liquid phase separation to have heterogeneous size pores ranging from 50 to 200 µm. Delivering RPCs on a PLA/PLGA composite graft .3 mm in thickness induced morphological changes in RPCs that were evidence of differentiation. The cells exhibited a high degree of polarization consistent with photoreceptor cells as well as an increase in cell survival. Attachment of RPCs to the scaffold was associated
with down-regulation of immature cell markers and up-regulation of mature cell markers.

1.4.2.2 MEMS-based 2-D Scaffolds

The concept of cell delivery via a scaffold was advanced further by integrating computer-aided design and microfabrication processing technology to control scaffold architecture, including parameters such as porosity and topography. MEMS technology allows for more precise control of scaffold features and more uniform, reproducible pore size and shape. The micropatterned substrates can be designed to encourage cell attachment, migration, and differentiation into the appropriate phenotype. Furthermore, the microfabrication processing allows for a scaffold design closest to native retinal anatomy and increases the potential for reformation of photoreceptor synapses. This technology has been applied to a variety of polymers to fabricate scaffolds for RPC grafting.

One study evaluated the effect of non-degradable poly(methyl methacrylate) (PMMA) scaffolds 6 μm thin on the survival, migration, and differentiation of RPCs.\textsuperscript{19} It showed that porous micropatterned PMMA scaffolds demonstrate enhanced RPC adhesion and cell migration into the host retina as compared to smooth, non-porous scaffolds. Photolithography and reactive ion etching were used to make an 11 μm pore diameter with 63 μm in between pores. The study showed that even though porous and non-porous scaffold exhibit biocompatibility, cell survival, and adherence, the porous scaffold allows for better attachment of cells up to four weeks after transplantation to the subretinal space. Furthermore, the pores provided a stimulus from migration into the native retinal layers. The non-porous scaffolds had limited retention of RPCs.

In another example of precisely designed pore structures, Neeley et al. fabricated a PGS scaffold for retinal progenitor cell grafting using standard microfabrication and replica molding techniques\textsuperscript{20} The scaffold, 45 μm in thickness, consisted of a uniform porous structure with 50 μm pores, approximately the thickness of the scaffold. It exhibited mechanical properties well suited for the
retina, including Young’s modulus (1.66 ± 0.23MPa) and maximum elongation at failure of 113 ± 22%. RPCs strongly adhered to the scaffold and proliferated. Immunohistochemistry revealed the presence of some mature cell markers at various time points after cell seeding, indicating cell differentiation. This study further demonstrated the ability of RPCs to attach to polymer scaffolds in the subretinal transplantation process as well as the potential of using a porous design. The cells are able to attach to the scaffold via cell anchorage mechanisms to the pores and embedment inside the pores. This also allows other surrounding RPCs to attach to the scaffold via cell-cell contact.

1.4.2.3 Nanostructured 2-D Scaffolds

Engineered nano-scale features on scaffolds made of fibrous or porous materials also have the ability to elicit a cell response. For example, nanowires have shown biocompatibility, cell adhesion and migration, and tissue organization. In one study, RPCs were cultured on smooth poly(e-caprolactone) (PCL) and on short and long biodegradable, thin film nanowire PCL scaffolds. PCL nanowires and nanofibers with average diameter of 150-100 μm, length of 2.5 μm or 27.5 μm, and a spacing of 20 μm, were fabricated using a hot melt templating approach. Not only are the nanowire scaffolds very thin, but PCL is also very permeable and has a predictable degradation rate well-suited for subretinal implantation. RPCs were seeded on these constructs, and the scaffolds were then transplanted into the subretinal space of mice for observation of RPC integration, differentiation, and long-term survival.

The RPCs seeded on the smooth PCL randomly adhered to the surface and remained spheroid in shape. By day 3, there was no alignment to specific surface regions, presumably due to the lack of structural cues, though there were cell-cell contacts. The long PCL nanofibers folded over to form microstructured ridges that the RPCs adhered to while remaining spheroid. The RPCs that localized to the formed micropits extended outwards to maintain contact with the ridges. Because the short, densely-packed nanowires reduced the surface area that cells could attach
too, RPCs attached individually to the tips of nanowire groups. On day 3, the cells appeared to have formed cell-to-cell fan-like processes thereby creating contacts. Furthermore, after day 7 the cells had formed a dense monlayer on the nanowire surfaces with processes connecting the cells. The RPCs proliferated strongly and expressed mature retinal proteins in response to the interactions with the nanowire scaffold. The cells also demonstrated significant migration ability. This study builds on other findings that RPCs have the potential to respond to the structural stimulus of micro- or nano-patterned surfaces. This can be used advantageously to direct the differentiation of cells. The cell-scaffold composite grafts improve the survivability and differentiation of the transplanted cells and have applications in retinal disease treatment.

1.4.3 Disadvantages of Current Strategies

There are a number of disadvantages of the methods described above, warranting the need for further development of practical solutions for stem cell delivery to the subretinal space. As described above, stem cell bolus injections have too many shear forces in the delivery process, thereby diminishing cell survival and proliferation. Thus, scaffolds are a necessary element of retinal regenerative therapy, but they should be able to carry a large number of cells and have an organized, defined structure. Bulk, porous scaffolds of various size pores can carry a large number of cells, but the cells are not able to migrate out of the scaffold easily. Cells are also distant from each other as well as from the host environment. These scaffolds are thick and may be disruptive to the native retinal tissue. Furthermore, they are not reproducible in design. Thin film 2-D platforms allow for enhanced cell migration out of the scaffold, but they are only able to carry a limited layer of cells. In addition, this layer can be sheared off relatively easily during the transplantation process. Clearly, there is a need for a precise, uniform device design that can hold a large number of cells and allow for cell survival and migration.
1.5 Biomaterials

1.5.1 Role of Biomaterials in Regenerative Therapy

Biomaterials play a fundamental role in the fields of regenerative medicine and tissue engineering. Interactions of cells, which are either transplanted in the scaffold or already in the host tissue, with the scaffold material determine how the cells function and respond upon transplantation. Biomaterials are also able to direct cell-cell interactions and guide cell responses. Both synthetic and natural polymers have distinct merit in applications, as do hybrid polymers. Furthermore, the scaffold material serves to strengthen the structural integrity of the defect site during the period of regeneration. It prevents infiltration or collapse of surrounding tissue into the defect site. The biocompatibility and mechanical compatibility of the scaffold material are central to the efficacy of a device. The 2-D and 3-D patterning of biomaterials can achieve a biomimetic model of complex tissue architecture. Finally, the biomaterial’s permeability and degradation rate directly impact the time course of tissue regeneration.

1.5.2 Existing Materials in Use

Despite advances in the field of biomaterials science and development of a variety of polymers, it is difficult to find a material with optimal mechanical properties for retinal tissue engineering and one that can be fabricated at the desired thinness for use in the retina. For example, though PLGA is biodegradable, it does not have the best material properties for a retinal implant. It has rigid and firm mechanical properties, exhibits bulk degradation, and has limited biocompatibility in some cases.22

Poly(glycerol sebacate) (PGS) is a tough biodegradable elastomeric polymer that has mechanical properties well-suited for tissue applications. It was first synthesized in 2002 by Y.Wang et al. based on glycerol and sebacic acid.23 The elastomer forms a covalently crosslinked, three-dimensional network of random coils. It shows good biocompatibility, is relatively inexpensive to synthesize, and is fairly flexible. This evaluation found that PGS degrades primarily by surface erosion.
in vivo, preserving geometry and retaining mechanical strength as mass decreases. 24 PGS has been studied as a material for tissue engineering scaffolds for applications such as cardiac, soft, and vascular tissue engineering. However, curing PGS to the desired thinness required for this retinal application and with facilitated delamination is challenging.

1.5.3 Suitability of Polycaprolactone (PCL) for Retinal Applications

Previous studies have shown that RPCs can be cultured with PCL nanowire substrates. 25 These scaffolds were biologically compatible with RPCs given the indications of cell adhesion and proliferation. Furthermore, the thin film structure of the PCL offered two important advantages for transplantation to the subretinal space. First, the thinness allows the scaffold to be placed with minimal disturbance to the host retinal tissue. Secondly, PCL is highly permeable and allows for physiologically significant molecules such as nutrients to pass through the scaffold. In addition, the scaffold has a predictable degradation rate; the process occurs gradually at the scaffold surface. As it degrades, there are no increases in local acidity as seen in bulk degradation of higher molecular weight polymers such as PLGA. 26 PCL is a degradable polyester with a low melting point around 60°C. The features of PCL are conducive to the efficacy of stem cell delivery to the subretinal space via a microfabricated scaffold.
Chapter 2: Specific Aims and Scaffold Design

2.1 Scaffold Design

Given the shortcomings of current retinal progenitor cell grafting techniques described above, there is a need for a retinal tissue engineering scaffold that meets the following criteria:

- Measures approximately 30μm in thickness to prevent harm to the native retina.
- Delivers and supports survival of a maximum number of cells during transplantation.
- Encourages migration of cells out of the scaffold.
- Prevents infiltration of surrounding cells and tissue.
- Has mechanical properties compatible with the retinal tissue and serves to reinforce retinal tissue architecture.

The proposed scaffold structure in Figure 2-1 is designed to accomplish these goals.

![Diagram of scaffold design](image)

**FIGURE 2-1:** Cross-section view of proposed scaffold design to show both interior and exterior.

In Figure 2-1, a 3 cage x 3 cage scaffold design is shown for clarity. A fabricated scaffold construct is a circle measuring 5mm in diameter, has approximately 300 cages to hold cells, and can hold approximately $2.6 \times 10^4$ cells in total. The scaffold
size can also be increased or decreased depending on the size of the biopsy punch used for fabrication. Figure 2-2 depicts the relative location of the device in the retina upon transplantation.

![Diagram](image)

**FIGURE 2-2:** Depicts location of the device in the retina post-transplantation.  

As shown in Figure 2-2, the device is inserted between the photoreceptor neuroretina and the retinal pigment epithelium via an incision by the surgeon through the front or the back of the eye. Figure 2-3 depicts the cross-section of the scaffold with the dimensions of an individual cage for cell encapsulation. The thickness of the entire scaffold is approximately 30 μm, which is thin enough to prevent serious damage to the host retina. Notable design measurements are the cage diameter of 216 μm, the nutrient infiltration pores with a diameter of 10 μm, and cell migration pores with a diameter of 72 μm.
FIGURE 2-3: Cross-section of the scaffold with dimensions of an individual cage for cell encapsulation (all units in μm). Each cage can hold approximately 88 tightly packed cells.

There are several components of the rationale for the scaffold design, outlined below.

_Ultrathin scaffold_:
The 30 μm thickness will increase the chances of RPCs receiving copious nutrients and decrease the chance of the scaffold disturbing the host retinal tissue.

_Nutrient Infiltration:_ The condition that cells should be within 200μm of a nutrient source\(^2^8\) will be met with this scaffold design due to the many channels for nutrient delivery.

_Delivery of a large number of cells to subretinal space:_ There is a need for a scaffold that can encapsulate and deliver a large number of cells, unlike current designs in which one layer of cells is seeded onto a scaffold and can be easily sheared off during transplantation. The proposed scaffold design will be able to hold a large number of cells upon transplantation to the subretinal space. In addition, the cages will provide a protective environment to prevent damage to the cells. Given a scaffold cage diameter is 216 μm, the cell density in each cage will be large enough to populate the subretinal space yet not too large so as to inhibit the cells from proliferating. Each cage is capable of holding 88 tightly packed cells by volume assuming cells are approximately spherical with a radius of 10 μm. A circular
scaffold measuring 5 mm in diameter has approximately 300 cages and can encapsulate approximately $2.6 \times 10^4$ cells total.

**Unidirectional cell migration out of the scaffold:** The device will not only allow the cells to migrate out of the scaffold but will also do so in a unidirectional manner. Due to the larger pore size on one end (72 μm) and smaller pore size on the other end (10 μm), the RPCs will selectively migrate unidirectionally towards the photoreceptors in the retina out of the larger pore because they are too large to travel across the smaller pores. Nutrients will diffuse in from the pigment epithelium through the smaller pores, which are 10 μm in diameter. This will allow for nutrient delivery into the channels while preventing cell migration out of the channels towards the pigment epithelium.

**Contact guidance cues:** The larger 72 μm pores for cell migration are designed with hexagonal ridges to serve as built-in contact guidance cues that promote the attachment and migration of cells. Contact guidance cues with ridge-groove geometries have been demonstrated to enhance cell orientation and morphology in previous literature. The shape of the posts on the silicon wafer negative mold that is used to synthesize the pores for cell migration out of the scaffold is shown in Figure 2-4.

![Figure 2-4: Shape of posts on silicon wafer mold that is used to synthesize cell migration pores with built-in contact guidance hexagonal ridges, shown in L-Edit design software.](image)
The hexagonally ridged geometry in the PCL scaffold will line the 10 μm thick cell escape pores and provide contact guidance. Not only will this encourage the cells to migrate out of the scaffold, but it will also do so unidirectionally since these ridges are only present on one end of the scaffold.

_Open to the retina on both sides:_ The scaffold is open to the retina on both sides. This will allow for endogenous nutrients and regulators to infiltrate the scaffold. In addition, it will allow for better integration of the scaffold into the subretinal space. Lastly, this feature makes the retinal regenerative process less dependent on the degradation rate of the PCL polymer because the tissue can already communicate from one end to the other.

_Large Surface Area:_ The large surface area created by the cages allows for more cell adhesion and migration ability.

_Potential for Drug delivery:_ This design can be readily modified to incorporate growth factors and other small molecules. This would allow the RPCs to begin differentiation prior to their migration out of the scaffold. By the time the cell reaches the photoreceptor layer, it will have received cues to differentiate into a photoreceptor cell and localize to that region of the retina. This will diminish the likelihood of the cell migrating elsewhere in the retina or differentiating into a different cell in the RPC lineage.

### 2.2 Advantages of the Scaffold Design

The scaffold design allows for delivery of a large number of cells contained in an organized, 3-D MEMS-based scaffold. The micropatterned uniform and precise architecture increases the anatomical organization of the device and increases the chances of developing functional photoreceptors and forming synapses. The scaffold design also enhances protection of the cells during the transplantation procedure. It is biodegradable and biocompatible, facilitating its transition to _in vivo_ testing and
human applications. The scaffold is open to the retina on both sides, allowing for communication, nutrient infiltration, and integration. The cells are centrally held, while channels on one side allow nutrient delivery from the retinal pigment epithelium and channels on the other side allow for RPC migration into the photoreceptor layer of the retina. The channels for cell migration have built-in contact guidance cues via hexagonally ridged pores in order to encourage cell migration out of the scaffold into the photoreceptor layer. In addition, this allows the cells to respond to the host environment without dependence on the degradation rate of the polymer.

Furthermore, scaffold parameters can be simply altered during fabrication to increase the number of cells the device can load. The channels that open to the retina can also be altered in terms of size and shape. The unidirectional migration of the cells is due to the sizing of the channels; the channel size is large enough for cells to migrate through on only one side of the cages. The thinness of the entire scaffold limits the invasiveness of the device on the host retinal tissue. Furthermore, due to the layer-by-layer construction of the device, each layer can be modified and tailored to meet specific topological, chemical, or drug delivery needs. Each layer can also have a different topography on either side of it for tailored interaction with the transplanted or host cells. Finally, the method is cost-effective and highly reproducible as a result of the photolithography and soft lithography techniques employed.

There are several novel features of the device that distinguish it from other retinal therapies in development. First is the design of an architectural chamber for encapsulation of cells and promotion of retinal differentiation. Second is the thin film three-dimensional nature of the scaffold that allows for communication of the interior of the device to the exterior on both sides. In addition, this decouples the cell integration and differentiation from the material degradation properties. Finally, the scaffold affords the opportunity to precisely control physiological, chemical, or structural features and enhance RPC culture.
Chapter 3: Fabrication of Polycaprolactone Thin Film 3-D Scaffolds

3.1 Introduction to MEMS

MEMS are micro-electro-mechanical systems and technology at the micro-level. MEMS technology can be implemented using a variety of materials and processing techniques. Materials include silicon, polymers, and metals. Processes include deposition, photolithography, etching, and micro-machining. Three-dimensional thin film scaffolds with features at the micron scale are fabricated using MEMS based microfabrication strategies in order to allow for precise control of the scaffold structure. Specific processes that will be used to fabricate the device elements include photolithography, standard replica molding, and soft lithography techniques. The overall fabrication process includes the following procedures: mask design using L-Edit software, standard SU-8 photolithography, standard replica molding, soft lithography with PDMS, polycaprolactone (PCL) thin film fabrication, and pressure bonding of thin films.

3.2 Materials and Methods

3.2.1 Mask design using L-Edit Software

 Masks for the three layers of the scaffold structure are designed using L-Edit Software. The L-Edit designs for the three individual scaffold layers are shown in Figure 3-1.

FIGURE 3-1: Designs of the three scaffold layers in L-Edit software. Left: nutrient pore layer, Middle: cell cage layer, Right: cell migration pore layer.
Feature layouts prepared in microfabrication design software (L-Edit) are printed onto transparent flexible photomasks using a laser printer with up to 20,000 dpi resolution (Art/CAD Services in photoplotting).

3.2.2 Standard SU-8 Photolithography

Standard photolithographic techniques are used to create the negative mold silicon masters for use in replica molding. Three separate silicon masters are made to fabricate three layers of the scaffold, which are then bonded together. These photomasks are used to transfer feature patterns onto 100mm silicon wafers patterned with SU-8 2000 series photoresist (Microchem) using a mask aligner (Karl Suss). The height of the SU-8 layer is tunable simply by choice of the specific SU-8 series product or the spin speed during SU-8 deposition on the wafers. The mask aligner exposes the photosensitive resist to UV light through the mask. During development of the resist, any areas exposed to UV light remain cross-linked to the silicon surface, while unexposed areas are washed away. Lithographic masters are postbaked and then passivated using a Teflon-like coating layer deposited in a reactive ion etcher (STS).

3.2.3 Replica Molding and Soft Lithography using PDMS

3.2.3.1 PDMS Synthesis

PDMS is synthesized by mixing PDMS prepolymer and curing agent in a 10:1 ratio (Sylgard 184, Dow Corning). The mixture is evacuated for about 15 minutes or until air bubbles are no longer present.

3.2.3.2 Replica Molding and Soft Lithography using PDMS

The PDMS is cast onto the silicon wafer in a petri dish in order to get a workable mold and then evacuated for 15 minutes. It is cured at 65°C for 2 hours, and then the mold is removed. This step is shown in Figure 3-2. These PDMS (-) molds are silane-coated overnight with 5 μl of silane. PDMS is then molded off of these silane-coated PDMS molds to make PDMS (+) molds for use in PCL spin casting. The PDMS (+) molds are replicas of the silicon wafers due to the double-inverse replica molding.
a. Synthesis of top layer (faces photoreceptor layer)

b. Synthesis of middle layer (encapsulates cells)

c. Synthesis of bottom layer (faces pigment epithelium)

**FIGURE 3-2:** Schematic of the first step of double-inverse replica molding with PDMS. This step generates the PDMS (-) molds that are then silane coated. This process is repeated with the silane coated molds to generate PDMS (+) molds, which are replicas of the silicon wafer.

### 3.2.4 PCL Thin Film Fabrication

#### 3.2.4.1 Polycaprolactone Synthesis

A 10% solution of high molecular weight (80,000 MW) polycaprolactone is dissolved in dichloromethane. The mixture is mixed for 3 hours at room temperature.
3.2.4.2 Polycaprolactone Spin Casting and Solvent Evaporation

The PDMS (+) molds are taped to silicon wafers with double-sided tape in order to facilitate spin-casting. 5ml of 2% soap solution (Micro-90 Concentrated Cleaning Solution, Cole-Parmer Instrument Company) is spun on the molds at 1500 RPM for 30 seconds to facilitate PCL release after spin-casting. 15ml PCL is poured on the molds and let spread for 15-20 seconds. Then, as shown in Table 3-1, the molds are spun at different speeds for 30 seconds depending on the mold's design in order to optimize the formation of through-pores. They are spun open to air in order to enhance solvent evaporation.

Table 3-1: Optimized Spin Speeds for PCL Spin-Casting

<table>
<thead>
<tr>
<th>PDMS Mold</th>
<th>Spin Speed (rpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient Pores</td>
<td>1500</td>
</tr>
<tr>
<td>Cell Cages</td>
<td>2200</td>
</tr>
<tr>
<td>Cell migration pores with ridges</td>
<td>2000</td>
</tr>
</tbody>
</table>

Then, the molds with spin cast PCL are placed in an oven at 70°C for 10 minutes to melt the PCL. This is done to encourage the formation of through-pores and enhancement of the design features by allowing PCL to infiltrate crevices of the molds. The molds are spun again to facilitate drying at 1500 RPM for 20 seconds. The PCL layers are then released from the molds either by submerging in water or by peeling with tweezers. All PDMS molds are rinsed with water or methylene chloride in between uses in order to remove any PCL still on the mold.

3.2.5 Pressure Bonding

Sections of each PCL thin film with through-pores, as evidenced by examination of the films under microscope, are cut using biopsy punches. As shown in Figure 3-3, the three unique layers of PCL are stacked in order from bottom to top- nutrient pores, cell cages, cell migration pores- and aligned with the aid of 70% ethanol to keep the sections from curling. The 3-D structure is held between glass slides and pressure bonded under vacuum in a laminator at 40°C for 20 minutes. A maximum of 3 scaffolds should be pressure bonded in the laminator at one time to ensure a uniformly patent pressure bonding of all samples.
3.3 Results

3.3.1 Individual PCL Thin film Scaffolds

All images are captured using scanning electron microscopy (SEM). Samples are cut and trimmed from thin film PCL scaffolds using a biopsy punch or razor blade. They are sputter coated for 30 seconds at 30mA current using a Cressington 108auto Automatic Sputter Coater. They are then imaged using a Hitachi S-3500N Scanning Electron Microscope.
Using the methods described, all three layers have regions of through pores shaped according to their respective designs. Figure 3-4 shows the relative size of the features of each layer in order to depict the size of the cages for cell encapsulation as well as the larger size of the cell migration pores as compared to the nutrient infiltration pores.

**FIGURE 3-4:** The relative sizes of the features of the three PCL layers. Left: nutrient pores, Middle: cell cages, Right: cell migration pores.

Figure 3-5 shows the thickness of the cell cages layer, which usually measures an average of 12 µm. The three layers range in thickness from 8 µm to 13 µm according to SEM measurements taken. Higher spin speeds yield a thinner PCL film. Usually, PCL made from the synthesis procedure described above and spun at 1500 RPM for 30 seconds forms a thin film approximately 10 µm thin.

**FIGURE 3-5:** The thickness of the cell cages layer.

Figure 3-6 shows zoomed-in images of the nutrient pores and cell migration pores. The nutrient pores exhibit relatively uniform circular geometry. The cell migration pores clearly have hexagonally spaced ridges or grooves. However, these features
are less uniform than other mask designs. This is likely due to the more complicated geometry and inability of PCL to fully mold around the shape of the posts.

**FIGURE 3-6:** A closer look at the smallest features of the scaffold. Left: nutrient pore layer, Right: a cell migration pore with contact guidance ridges.

In Figure 3-7, the top of the PCL thin film that interfaces with the air during spin-casting and the bottom that interfaces with the PDMS are shown. Clearly, the top results in a peak thickness around the posts while the bottom has flat topography.

**FIGURE 3-7:** Surface topography of both sides of the nutrient pore layer. Left: the top side during the spin-casting process results in formation of a peak thickness around the PDMS posts. Right: the bottom side that is against the PDMS during the spin casting reveals a flat surface.

Figure 3-8 depicts the bendable nature of the polymer as well as the crystalline lattices of PCL thin films. The bendable properties of the scaffold are optimal for this
retinal application because the device can better assume the landscape of the subretinal space.

![Image of cell cages and cell ridges scaffolds](image)

**FIGURE 3-8**: Texture and bendable properties of the PCL thin films. Left: The cell cages scaffold shows the crystalline geometry of cured PCL. Right: The cell ridges scaffold demonstrates the ability of PCL to deform and bend.

Table 3-2 summarizes the average diameter and standard deviations of design features at sequential steps in the fabrication process: software designs, transparency masks, SU-8 photolithography, and PCL spin-casting. Each value is an average of five points (center, top, bottom, left, right) measured in a region visualized to have through-pores under a microscope. Table 3-3 summarizes the percent difference in feature diameter between the initial software design and the final PCL thin film.

### Table 3-2: Average Size of Design Features

<table>
<thead>
<tr>
<th></th>
<th>L-Edit Software Design (µm)</th>
<th>Transparency Mask (µm)</th>
<th>Silicon Wafer (µm)</th>
<th>PCL thin film (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient pores</td>
<td>10</td>
<td>10.1 ± .15</td>
<td>12.0 ± .34</td>
<td>11.8 ± .80</td>
</tr>
<tr>
<td>Cell Cages</td>
<td>216</td>
<td>217.5 ± .44</td>
<td>218.8 ± .90</td>
<td>213.4 ± 1.8</td>
</tr>
<tr>
<td>Cell migration pores (including ridges)</td>
<td>72</td>
<td>73.0 ± .44</td>
<td>65.3 ± 1.0</td>
<td>62.7 ± 2.1</td>
</tr>
</tbody>
</table>

Note: ± denotes standard deviation
### Table 3-3: Percent Difference in Feature Diameter Between PCL Scaffolds and L-Edit Software Design

<table>
<thead>
<tr>
<th>Feature Type</th>
<th>% Difference (PCL relative to L-Edit design baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient pores</td>
<td>+18.0</td>
</tr>
<tr>
<td>Cell Cages</td>
<td>-1.2</td>
</tr>
<tr>
<td>Cell migration pores (including ridges)</td>
<td>-13.0</td>
</tr>
</tbody>
</table>

#### 3.3.2 Three-Dimensional PCL Scaffold

The three individual layers are pressure bonded under vacuum to form a patent 3-D thin film scaffolds. SEM images from various angles of the scaffold are shown in figure 3-9. The top left image shows the edge of a stacked and bonded cage scaffold with the nutrient pores facing upwards. The edges of the cell cages are visible through the nutrient pore layer. In the top right image, which shows the scaffold from the top, the edge of the cell cage, contact guidance pores, and nutrient pores are all visible. The bottom left image shows a magnification of one cell cage of the stacked and bonded scaffold with the contact guidance pores facing up. The variations in the shape of the cell migration pores with contact guidance ridges are visible in this view. Finally, the bottom right image shows the scaffold magnified on one contact guidance pore, showing all three layers.
FIGURE 3-9: Image of the 3-D scaffold after pressure bonding. Top left: view from the back showing nutrient pores. Top right: View from the top of the scaffold. Bottom left: View from the front of one cage. Bottom right: View from the front of one cell migration pore.

3.4 Discussion

The fabrication process demonstrates that microstructured PCL thin films can be successfully fabricated using photolithography and soft lithography techniques. Furthermore, thin film scaffolds can be stacked and bonded to form 3-D scaffolds with a unique architecture. The methods developed are effective at fabricating the layers of the scaffold design and bonding them together to form ultrathin devices approximately 30 μm thin.
3.4.1 Precision of the Fabrication Process

The precision of the fabrication process from step to step can be gauged by the diameter of design features. As summarized in Table 3-2, the diameter of the features on all three layers varied after each step, as would be expected with features at the micron scale. The features designed in L-Edit software are all uniform in diameter. After conversion to transparency photomasks, all features exhibit a slightly larger diameter on average: nutrient pores are 0.1 μm larger, the cell cages are 1.5 μm larger, and the cell migration pores are 1 μm larger. These increases are attributable to the photoplotting software.

After the SU-8 photolithography process to convert transparency photomasks to silicon wafers, the nutrient pores and cell cages are larger in diameter by 1.9 μm and 1.3 μm, respectively. This may be a result of the exposure of a larger feature area of photosensitive resist to UV light through the mask than would be predicted by the design on the transparency. This wider exposure at each feature would crosslink a larger area to the silicon surface, resulting in a larger feature size. On the other hand, the average diameter of the cell migration pore decreases after this step by 7.7 μm, a substantial fraction of the feature. This may be a result of the more complicated geometry of the hexagonally ridged pore resulting in less feature area of photosensitive resist being exposed to UV light through the mask. This would result in a smaller feature size.

After soft lithography and double-inverse replica molding with PDMS, the diameter of features in the PCL scaffolds are reduced on average by 0.2 μm for the nutrient pores, 5.4 μm for the cell cages, and 2.6 μm for the contact guidance pores. It is possible that when the PDMS (+) mold that is a replica of the silicon wafer is being molded off of the PDMS (-) mold, the PDMS does not infiltrate the crevices of the PDMS (-) mold to the greatest extent possible. This may be a result of the silane coating on the PDMS (-) mold.

These acute variations in diameter are expected when working with such precise features at the micron scale. The variations are more significant for the smaller features because they are distorted by a larger percent of their original area,
depending on the magnitude of the variation. As summarized in Table 3-3, the percent difference in feature diameter between the original L-Edit design and the PCL scaffold is greatest for the smallest features, the nutrient pore layer. The next largest percent difference is for the next smallest feature, the cell migration pores. The design with the least percent difference between the L-Edit template and the PCL thin film is the cell cages, due to its larger design.

Furthermore, at every step, the standard deviation increased for all three layers. This increase in amount of feature variability is due to the fact that the process became increasingly user and materials dependent. The process becomes less precision-controlled by software and micromachining, resulting in greater variation. In addition, the cell migration pore with hexagonally ridged geometry exhibited the greatest standard deviations throughout the process. Again, this is likely attributable to the more complicated geometry of the design as compared to simple circle pores on the other two designs. The hexagonal ridges are capable of bending, twisting, and stretching, thereby increasing variation.

3.4.2 Reproducibility of the Process

The fabrication process is controlled and reproducible for future applications. However, the step with the greatest variation is the PCL spin-casting and solvent evaporation. Though every iteration for each layer of the scaffold yields regions of the PCL thin film with through-pores, the area of these regions varies. This variation determines the size of the 3-D scaffold that it is possible to fabricate. The most common size scaffold to fabricate is 5mm x 5mm because of readily available regions of this size with through-pores on each scaffold layer. Larger scaffolds such as 12mm x 12mm are possible but are fabricated less frequently, limited most often by the formation of through-pores on the cell migration pores layer. As a result of the more complex geometry of this design, there is greater variability of through-pore regions on this PCL thin film. The nutrient pores and cell cages are highly consistent in their outcome of a large area of through-pores in the PCL thin film.
Chapter 4: In Vitro Testing

4.1 Introduction

Medical devices are first tested in a controlled environment outside of organisms and animals in order to ensure that it will function as it is designed to. Devices made from biomedical engineering techniques are tested in an environment that simulates that of humans but does not have complications that may arise in humans. Thus, it is a first pass study to discern basic functionality of the device without the variables that traditionally arise in animals. The \textit{in vitro} experimentation of this microfabricated 3-D stem cell delivery scaffold will include seeding of the scaffolds with retinal progenitor cells (RPCs), evaluation of cell localization and migration in the scaffold, and an assessment of cell population in the cages designed for cell encapsulation.

4.2 Materials and Methods

4.2.1 Retinal Progenitor Cells

Cell type, scaffold material, and growth factors must be properly selected in order to best allow the cells to integrate in the host tissue and differentiate into photoreceptor cells. Previous research has shown that differentiated tissue is not able to integrate properly into host retinal tissue.\textsuperscript{30} In addition, neural progenitor cells do not completely differentiate into the desired retinal cells.\textsuperscript{31} Research has shown that retinal progenitor cells (RPCs) would be an effective cell type for retinal tissue engineering. They have shown an ability to integrate into the layers of the retina and are able to undergo complete differentiation into retinal neurons.\textsuperscript{32} As shown in Figure 4-1, RPCs form clusters of cells called neurospheres with increased cell growth and proliferation in suspension. In addition, RPCs are an immunopriveleged cell type and will not elicit an immunogenic response when injected into a subretinal space.\textsuperscript{33} RPCs are therefore an efficacious cell type to employ in this project.
4.2.2 Cell Seeding

Four 5mm x 5mm circular PCL scaffolds are prepared for cell seeding. The scaffolds are placed in a 24-well plate and sterilized in UV light for 55 minutes. The scaffolds are further sterilized by submersion in 70% ethanol for 2 hours 25 minutes. The scaffolds are rinsed 4 x 15 minutes in HBSS. They are then submerged in NB complete media at 37°C overnight.

Retinal progenitor cells are isolated and cultured at Schepens Eye Research Institute, Boston, Massachusetts. 1ml of cells at 4x10^5 cells/ml are spun down in a centrifuge at 1000 RPM for 7 minutes. The media is aspirated, and the pellet is resuspended in 500μl NB complete media as a wash step. The suspension is spun again at 1000 RPM for 6 minutes. The media is aspirated, and the pellet is resuspended in 40μl to make 1x10^7 cells/ml.

Two scaffolds are placed in a 96-well plate; their orientation was checked under a microscope to ensure the cell migration pores were facing up. 5μl of cell suspension is pipetted onto the scaffolds. The 96-well plate is placed in a vacuum desiccator. A weight is placed on the lid to prevent it from lifting off when the vacuum is pulled. Vacuum is pulled 6 times for 30 seconds each time at 4 in.Hg, releasing to atmosphere between each cycle, according to prior literature. The other two scaffolds are placed in the 96-well plate. They are directly seeded without vacuum backfill by pipetting 5μl cells directly on top of the scaffolds. The scaffolds...
are incubated for 45 minutes at 37°C before collecting Day 0 images by fluorescent microscopy. Then 60μl of media is added to each well and incubated overnight. 24 hours later, 50μl of media is added to each well. On days 3, 5, and 7, the majority of the media is pipetted out to flatten the scaffold and facilitate imaging. After imaging, 100μl media is added to each well and returned to the incubator at 37°C for another 48 hours.

The fluorescent microscopy images are analyzed using ImageJ software made available by the NIH. Images from Day 0, 3, 5 and 7 of the four experimental scaffolds are analyzed. It is assumed that any area of fluorescence indicates cell presence at that location. Therefore, fluorescence is an indicator of cell survival, proliferation, and localization. The area of RPCs GFP fluorescence in individual cell cages was measured cage-by-cage using the ImageJ area measurement software. Using the shape tools, the region of fluorescence of each cell or group of cells is defined by the user, and the software calculates the area of that region. An example of this method is shown in Figure 4-2.

![FIGURE 4-2: Selection of areas of cell fluorescence in ImageJ. The area of fluorescence in each cage is an indicator of cell presence, survival, and proliferation.](image)

Only cages that were both in focus and included the entire area of the cage were included for analysis. In addition, only cells inside the cages or at the border of the cages were counted. The area of fluorescence was converted to 'percent area of the cell cage covered by cells' by a simple calculation, using the approximate area of each cage as 3.53 x 10^4 μm². There was variability of the number of cages measured
for each sample at each time point. To keep the sample size the same and include cages with pertinent cell presence, the ten highest values of 'percentage area of cell cage covered by cells' for each scaffold at each time point were averaged.

4.3 Results

4.3.1 Retinal Progenitor Cell Localization

On day 7 after cell seeding, scaffolds are stained with 4 μM Ethidium solution (Invitrogen) for 30 minutes at room temperature and covered by foil. They are imaged using fluorescent microscopy with a rhodamine filter. This provides information about cell localization and efficacy of the standard cell seeding, Figure 4-3, versus the vacuum backfill seeding technique, Figure 4-4.

FIGURE 4-3: Ethidium staining on day 7 of the standard cell seeding scaffolds. It shows a random distribution of RPCs in relation to the scaffold features. Left: bright field image showing scaffold structure, Right: fluorescent image showing cell localization in relation to scaffold structure.

FIGURE 4-4: Ethidium staining on day 7 of the vacuum backfilled scaffold. It shows preferential localization of RPCs to the cages. Left: bright field image showing scaffold structure, Right: fluorescent image showing cell localization in relation to scaffold structure.
4.3.2 Confocal Microscopy

As shown in Figures 4-5 and 4-6, images taken by confocal microscopy on day 7 after cell seeding shed further insight on cell localization in the Z-direction. It is important to verify that cells are in fact in the middle layer of the scaffold in the cell cages and not merely seeded on top of the scaffold over the cell cages.

**FIGURE 4-5:** Confocal images of standard cell seeded scaffolds on day 7. The orthogonal view reveals that the cell at the intersection of the x- and y- crosshairs is in the middle of the z-stack. Over the time course, the cells were able to adhere to the scaffold and migrate into the cages.

**FIGURE 4-6:** Confocal images of vacuum backfilled scaffolds on day 7 after seeding. The orthogonal view reveals that the cell at the intersection of the x- and y- crosshairs is in the middle of the z-stack.
From fluorescent microscopy images, RPCs appeared to localize to regions of the cell cages over time, but the confocal images confirm that the cells are centrally located in the middle of the scaffold thickness. The green color in the confocal images surrounding the cell cages is merely reflection off of the PCL polymer or media during confocal imaging and not the presence of cells.

4.4.3 Cell Population in Cell Cages

As described above, the cell population in the cell cages is measured by calculating the area of RPC-GFP fluorescence in individual cages with ImageJ software. The graph in Figure 4-7 is generated using the average data of the two vacuum backfilled scaffolds and the average data of the two standard seeded scaffolds. The vacuum backfilled sample has a higher initial cell population in the cages. However, the RPC population in these scaffolds begins to fall off sooner than the population in the standard seeded scaffolds.

**FIGURE 4-7:** Measurement of cell population in cell cages by % area of cell fluorescence over a 7 day time course.
4.4 Discussion

4.4.1 Scaffold Integrity during Cell Culture

As demonstrated in previous literature, these in vitro experiments confirmed that PCL is a polymer compatible with RPC applications. The RPCs can be cultured in the scaffold, and they would be able to achieve greater cell densities with delivery of optimal therapeutics. Furthermore, the imaging showed that the scaffold is patent throughout the 7 day time course in the experiment. The individual layers do not come apart or disintegrate over the period of the cell culture. The pressure bonding technique used to bond the layers is clearly sufficient for this application. The 3-D scaffold is an effective device to encapsulate and culture cells.

4.4.2 Cell Localization and Migration

As revealed by GFP fluorescence in images captured by fluorescent and confocal microscopy, the cells are able to localize to the cages designed for cell encapsulation. However, the vacuum backfill seeding technique results in greater localization in the cages, while the standard cell seeding technique results in a more random distribution of cells. Seeding technique is important to consider given that the goal of the procedure is to optimize cell encapsulation specifically inside the cages. In Figure 4-4, a scaffold that was seeded using vacuum backfilling, the majority of cells appear to be contained specifically in the cages. On the other hand, in Figure 4-3, a scaffold that was seeded using standard techniques, the cells appear to more randomly distributed and less localized selectively to the cages. There appear to be cells in between cages, perhaps on top of the scaffold.

Confocal microscopy is necessary to ensure that the cells actually localize inside the cages and not simply on top. Using stacks in the Z-direction, the specific location within the 3-D scaffold can be determined. As shown in Figure 4-6, scaffolds seeded by vacuum backfilling, alignment of the crosshairs on a specific cell shows the location of the cell in the z-stack in the orthogonal view. In both examples, the cells are in the center of the scaffold in the z-direction. This is the location of the centrally located cages for cell encapsulation. In Figure 4-5, scaffolds seeded by
standard techniques, cells are also centrally located in the z-direction, indicating their containment inside the cages. These cells were able to migrate through the hexagonally ridged pores and into the cages over the 7 day time course. This is also a strong indication of the migration ability of RPCs through the hexagonally ridged pores with built-in contact guidance cues. In order for the cells to travel from the top of the scaffold after standard cell seeding to the inside of the cages, they must have had to adhere and migrate. This indicates that the hexagonal ridges pores will effectively allow for cell migration out of the scaffold into the subretinal space after transplantation.

4.4.3 Cell Survival

The measurement of cell population in the cell cages by average percent area of the cell cage covered by fluorescence sheds light on cell survival and proliferation over the 7 day time course. It also allows for a comparison of the vacuum backfill seeding technique to the standard cell seeding technique. According to Figure 4-7, the vacuum backfilled scaffolds have more than twice the initial cell population in the cages than the standard cell seeded scaffolds. This reaffirms the findings in the fluorescent microscopy images that the vacuum backfill technique leads to more selective RPC localization to the inside of the cages. Furthermore, the vacuum filled scaffolds have higher cell populations for the duration of the time course. To increase the RPC population in the cages and reduce the number of seeded cells that are not ultimately encapsulated by the cages, the vacuum backfill technique is likely the stronger the choice.

Both scaffold types exhibited steady rises in the presence of cells in the cages, but the duration of this rise differed. For the vacuum backfilled scaffolds, the cell population increased for the first five days but was tapering off by day 7. On the other hand, the cell population in the standard seeded scaffolds continued to increase over the 7 day time course. According to this data, even though the vacuum seeded scaffolds have higher initial cell populations in the cages, the cell viability falls off sooner than the standard seeded scaffolds. This may be a result of the
vacuum backfilling process. It is possible that the technique exerts forces on the RPCs that reduce their survival chances even as they localize to the cages. Though the standard seeded scaffolds have a lower initial cell population, they exhibited continued proliferation over the 7 day time course.

These results deem it difficult to conclusively determine the duration of cell viability in these scaffolds or the cell seeding technique that allows for greater cell viability, if any. Further studies with a larger number of scaffolds and sacrificial scaffolds for testing at individual time points is needed for more definitive results about cell survival and proliferation. However, it does show promise that all scaffolds showed an early boost in cell population, despite the varied duration of the boost. Further studies should be geared towards optimizing and lengthening the duration of the cell proliferation period.
Chapter 5: Conclusion

5.1 Overview of Current Progress

There is a need for improved treatment options for retinal diseases such as AMD and RP that degenerate photoreceptor cells and ultimately leads to blindness. The goal of this thesis project was to engineer a scaffold for retinal progenitor cell grafting using standard microfabrication techniques. The specific aims are to design the scaffold architecture, fabricate it using photolithography, soft lithography, and replica molding techniques, and evaluate the scaffold \textit{in vitro} using retinal progenitor cells. The scaffold offers several advantages that previously designed scaffolds for retinal progenitor cell grafting do not offer. It is able to deliver a large number of cells without the possibility of the cells being sheared off. It allows for unidirectional migration of cells out of the scaffold and into the subretinal space. It is ultrathin and open to the retina on both sides, allowing for nutrient infiltration. The scaffold can serve as an organized framework to support cell migration and integration, a matrix to promote cell adhesion, a barrier to prevent infiltration of surrounding tissue, a reinforcement of the retinal tissue structure, and even a delivery vehicle for growth factors.

This cell-scaffold composite will allow for controlled, precise, localized delivery of cells to the subretinal space to restore retinal tissue damaged by disease or trauma. Using precisely tuned structural, chemical, and physiological cues, retinal regenerative therapy and differentiation of a large number of photoreceptors for clinical use is distinctly possible. The culture of retinal progenitor cells in an environment that resembles the native retina can lead to redevelopment of degenerated retinal tissue. Due to the small size-scale of the retina and the need for fine-tuning of device features, microfabrication techniques are effective for retinal applications. With the aid of these techniques, the individual layers of the scaffold are patently bonded and the 3-D stem cell delivery scaffold for retinal regenerative therapy is fabricated. Furthermore, \textit{in vitro} experimentation confirms that RPCs can be cultured and delivered using this device.
5.2 Future Directions

Future in vitro testing includes experimentation with large numbers of 3-D scaffolds to allow for sacrifice of scaffolds at various time points. This will provide further insight into RPC viability and proliferation over a time course. The sacrifice of scaffolds will allow for use of diagnostics such as flow cytometry, plate reading, incremental live/dead assays, and various other staining techniques. Another step in the in vitro studies will include immunohistochemistry to determine expression of mature cell markers and provide an assessment of differentiation.

The device itself can be modified into a drug delivery vehicle. Incorporation of therapeutics by absorption, embedding, encapsulation, nanoparticle incorporation, or hydrogel delivery are all possibilities. These therapeutic molecules can initiate differentiation of the transplanted cells prior to migration into the neuroretina. Depending on which layer of the device is modified, therapeutics can be delivered to either the transplanted cells in the cages, the retinal pigment epithelium, or the photoreceptors in the neuroretina. Therapeutics delivered to the transplanted cells could enhance survival and differentiation. Molecules delivered to the retinal pigment epithelium could encourage angiogenesis. Therapeutics to the photoreceptors could help protect them from further degeneration associated with AMD or RP or from damage due to the implanted device.

Due to the physical inactivity and relative stability of the retina, mechanical testing is not crucial. However, testing can be done to determine whether pressure bonding, bonding via plasma treatment, chemical bonding, or thermal bonding enhances device strength. It would also be interesting to determine how PCL properties or the scaffold's architecture can be altered to best mimic the native retina.

In the in vitro studies, cell seeding was conducted via vacuum backfilling or conventional seeding techniques. Another approach to assess is cell encapsulation with a secondary gel matrix. The gel can provide an environment for the cells that is most conducive to neural differentiation irrespective of the material properties. Furthermore, the device design itself can be extended to other biodegradable
polymers, such as poly(glycerol sebacate) (PGS). The device can be made using other methods of fabrication such as sacrificial molding, injection molding, material printing, and laser machining.
References


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