ABSTRACT

FABRICATION AND DEVELOPMENT OF A PCL ELECTROSPUN FIBER-KERATIN AEROGEL SCAFFOLD TO MIMIC BRUCH’S MEMBRANE FOR THE STUDY OF AGE-RELATED MACULAR DEGENERATION

by Ziqian Zeng

Age-related Macular Degeneration (AMD) is a retinal disease responsible for 8.7% of blindness globally, and it is predicted that 196 million people will be affected by the year 2020. Further, there is currently no cure for AMD. Early stage AMD is usually triggered by dysfunction of the retinal pigment epithelium (RPE), and the Bruch’s membrane is often invaded by abnormal angiogenesis around the retina as the disease progress. A biomimetic tissue engineered retinal scaffold may be beneficial in understanding disease etiology and also a possible restorative treatment for AMD. As a primary step toward the development of a tissue engineered retinal scaffold, this thesis focuses on the conceptualization, design, fabrication, and characterization of a scaffold system that combines electrospun polycaprolactone (PCL) fibers to mimic the Bruch’s membrane and keratin aerogel to provide structural support reminiscent of the retinal choroid. Results of this work demonstrate that PCL/keratin fibrous-aerogel shows morphological similarity to the native retinal Bruch’s membrane under SEM, tunable compressive modulus within a range similar to the native retinal extracellular matrix, and minimal toxicity to ARPE-19 cell line. Importantly, the formation of cell monolayers suggests that such system may be suitable as a model system for testing key parameters associated with retinal injury and/or regeneration.
FABRICATION AND DEVELOPMENT OF A PCL ELECTROSPUN FIBER-KERATIN AEROGEL SCAFFOLD TO MIMIC BRUCH’S MEMBRANE FOR THE STUDY OF AGE-RELATED MACULAR DEGENERATION

A Thesis

Submitted to the
Faculty of Miami University
In Partial fulfillment of
The requirements for the degree of
Master of Science
Department of Chemical, Paper and Biomedical Engineering

by
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2017

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>RPE</td>
<td>Retinal pigmented epithelium</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>AMD</td>
<td>Age-related macular degeneration</td>
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<tr>
<td>GA</td>
<td>Geographic atrophy</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>PEDF</td>
<td>Pigment epithelium-derived factor</td>
</tr>
<tr>
<td>AREDS</td>
<td>Age-related eye disease study</td>
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<tr>
<td>PDT</td>
<td>Photodynamic therapy</td>
</tr>
<tr>
<td>LP</td>
<td>Laser photocoagulation</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly (caprolactone)</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>MTS</td>
<td>(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)</td>
</tr>
<tr>
<td>PMS</td>
<td>Phenazine methosulfate</td>
</tr>
<tr>
<td>hESC</td>
<td>Human embryonic stem cell</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
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<tr>
<td>HSD</td>
<td>Honestly significant difference</td>
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<tr>
<td>w/v</td>
<td>weight per volume</td>
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<tr>
<td>v/v</td>
<td>volume per volume</td>
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Acknowledgements

I would like to express my deepest gratitude to all the support I received from the Chemical, Paper and Biomedical department. This thesis work on electrospinning PCL fibers onto keratin aerogel for a scaffold useful for the study of Age-related Macular Degeneration is my first biomedical engineering research project. I have never enjoyed and dedicated myself so much in working on one project. And thus, I would like to thank my academic advisor, Dr. Justin M. Saul who had offered me this great opportunity in working with him and doing this challenging and independent research since the summer of 2015. And thank you for supporting, teaching and inspiring me with your expertise on biomaterials, tissue engineering, and drug delivery themes as well as all the analytical techniques from my Undergraduate Summer Scholarship (USS) program in 2015, to undergraduate senior design and Undergraduate Research Award (URA) in 2016 and all the way to my master degree thesis research. Thank you again for your support on the poster presentation at the BMES 2016 Annual Conference.

Also, I would like to thank my committee members, Dr. Coffin W. Douglas and Dr. Michael L. Robinson for the inspirations and suggestions towards the betterment of my project. I want to give my gratitude to Dr. Jessica Sparks for helping me with finding the way to fit compression testing model and Dr. Shashi Lalvani for introducing me to my current academic advisor.

Then, I also want to thank all people who work in the lab. Thank you to Judy Strassburger-Bohnert who taught me hydrogel fabrication. Thank you to Lam Phuong and Christian Gutierrez for supporting me with the ARPE-19 cells. Thank you to my senior design team members for solving the PCL solution issue.

At last, I want to thank my family for being my everlasting support.
CHAPTER I - Introduction to Age-Related Macular Degeneration and Electrospinning

Ziqian Zeng
I.1. Basic Anatomy of Retina

The focus of this thesis work is on the development of a biomaterial scaffold system that could be used to investigate key aspects of the retina or, ultimately, to be used in the treatment of Age-Related Macular Degeneration (AMD). In order to understand the design of the scaffold system, it is first important to understand the basic anatomy and physiology of the retina.

The retina is a tissue located at the posterior of the human eye [1]. Complete retina anatomy consists of neurosensory retina, retinal pigment epithelium (RPE) and the Bruch’s membrane. The macula is an oval shaped pigmented area near the center of retina. At the center of macula, there is a pit called fovea which is responsible for sharp central vision. This structure is summarized in Figure 1.

The vascular structure surrounding the Bruch’s membrane is called choroid. Choriocapillaris, the major component of choroid, is segmentally arranged in a single layer adjacent to the Bruch’s membrane and it acts as the major source for providing nutrients and oxygen to the retina.

Photoreceptors, the main cell type in the bottom layers of neurosensory retina, are specialized neurons that are responsible for converting incoming light into neurological signals for the brain to interpret, and this conversion process is also known as phototransduction [2]. In the photoreceptor layer, rods and cones are two cell sub-types that work specifically in phototransduction. In native retina, most photoreceptors in the inner layers are rich in mitochondria. The reason behind this is that phototransduction is an energy-consuming process that requires sufficient numbers of mitochondria and the subsequent energy generation that they can facilitate, especially when eyes dealing with vision in the dark. In fact, the retina is one of the most metabolically active tissues in animals [3]. Furthermore, as the energy generation process requires a large amount of oxygen inflow in the retinal tissue, the amount of molecular oxygen transport from choroid to photoreceptors becomes the main criteria to assure the quality of phototransduction.

Retinal pigment epithelium (RPE) is a monolayer of hexagonal cells firmly attached to the overlying photoreceptors and the underlying Bruch’s membrane. In vertebrate retina, RPE plays a multifunctional role [4]. By its unique location, RPE shapes a barrier between blood and retinal tissue to prevent unnecessary diffusion and cell migration. Moreover, this RPE barrier can help maintain a proper hydration level between choroid and neurosensory retina so as to offer good
visual clarity. As a part of the inner surface of the rear eye, RPE helps in absorbing stray light to ensure a better visual experience and this behavior also plays a role in tightening the retinal tissue connection to the choroid to maintain the retina’s structural integrity.

The Bruch’s membrane is a 2-4 µm thin connective tissue that comprises five layers: the RPE basement membrane, the inner collagenous zone, the zone of elastic fibers, the outer collagenous zone and the basement membrane of the choriocapillaris [5]. RPE basement membrane, which is also called the RPE basal lamina, is a mesh of fibers that accounts for 0.15 µm of the total RPE basement membrane; the thickness of the rest layers are 1.4 µm, 0.8 µm, 1-5 µm, and 0.07 µm, respectively, and their content is mostly fibers and vesicles [6]. As an extracellular matrix (ECM) complex, Bruch’s membrane offers many benefits to the surrounding cells, including physical support for RPE cell adhesion and regulation of molecular diffusion (e.g., of oxygen, nutrients, and waste) among layers [7].
Figure 1 Retina Anatomy.

This figure is reprinted by permission from Macmillan Publishers Ltd: [8] Nature Medicine, Forrester, J. V. "Macrophages eyed in macular degeneration." Nature medicine 9.11 (2003): 1350-1351, copyright (2017). The macula refers to the area at the center of the retina. Fovea is the central pit of the macula that is responsible for sharp central vision. The retinal pigment epithelium (RPE) is sandwiched between a basement membrane called Bruch’s membrane and the photoreceptors. Photoreceptors contain rods and cones, essential for phototransduction. The choroid is underneath the Bruch’s membrane, the dendritic cells and resident macrophages are essential for waste removal from the retina.
I.2. Age-Related Macular Degeneration and Prevalence

Age-Related Macular Degeneration (AMD) is a disease primarily related to aging. AMD typically becomes worse over time and can result in blindness once the disease progresses into its advanced stages. Patients with early stage AMD can be diagnosed with large yellow deposits or drusen under their retina. Progression into advanced AMD will cause central vision loss or even blindness. This corresponding central area that accounts for the vision loss in the eye is a small part of the retina referred to as the macula.

AMD affects millions of people, and it is one of the leading causes of blindness that is responsible for 8.7% of all blindness worldwide [9]. It is predicted that by 2020 around 196 million people globally will have some form of AMD [10]. A study by Friedman, et al. indicated that the prevalence of AMD in older age (over 65 years old) people is higher than those of younger ages (under 65 years old). Pooled data from eye surveys and studies in the United States indicate that very large increases in disease incidence rates were observed for both older males and females. For example, a comparison of female European-descended people of over 80 years of age with those of 50-60 years of age indicates that the AMD patients rate in the older population is 80 times larger than the younger age group. The current total advanced AMD population in the U.S. is estimated to be 1.75 million and it is believed that due to the rapidly aging population, the number will sooner double [11-13]. A disease and depression relationship study [14] indicted that among all individuals suffering vision loss due to advanced AMD, over half of them had reported significant declines in discretionary activities and around 30% had reported different levels of depression. From these studies, it is clear that AMD is already a significant problem in the U.S., that the burden will increase, and that this disease has both physical and psychological aspects.

From a pathology standpoint, early stages of AMD are known by several names which include early/intermediate AMD, early AMD, or early dry AMD. These stages are distinguished from advanced AMD. Early AMD is characterized by the size of drusen, a buildup of cell debris and lipids derived from the incomplete breakdown components of photoreceptor outer segments that are then expelled by the RPE within and around the Bruch’s membrane [15]. This buildup of lipids around the Bruch’s membrane impedes the ability for small molecules to diffuse between the Bruch’s Membrane and the choroid. Dysfunctions of the RPE as well as choriocapillaris atrophy are prominent symptoms of early/intermediate AMD. Accumulation of cell debris and other molecules intended for transport away from the eye cause local atrophy around the Bruch’s
membrane [16]. However, several small regions of drusen will cause little or no damage to vision. Actually, minor and small drusen formation is considered to be very normal among people over 50 years old [17]. Further, individuals with small regions of drusens can control progression by lifestyle changes such as improved diet [18]. But, larger drusen (diameter larger than 125µm) is considered to be severe and leads to risk of disease progression, which may further lead to severe central vision loss [15-16]. According to Wisconsin age-related maculopathy grading system [19], early AMD was strictly defined as either large drusen (diameter 125 µm to 249 µm) or any size drusen with pigmentary abnormalities. From the estimation by the Eye Disease Prevalence Research Group, over 7 million people in the U.S. have large drusen (>125 µm) in at least one eye, and it is believed that those cases had a potential risk of progression into advanced AMD over 5 years [13].

As opposed to early-stage AMD, advanced AMD is often associated with vision loss [18-22]. Based upon its pathological features (Figure 2), advanced AMD comprises two forms: geographic atrophy (GA), also known as “late dry AMD”, and neovascular (or exudative) AMD, also known as “wet AMD”. The formation of GA AMD is contributed to by local atrophy in the Bruch’s membrane as a result of RPE death and accumulation of cell debris. Since early AMD and GA AMD are all non-exudative, meaning no fluid leaking, they are both categorized as “Dry AMD.” In literature reviews, many studies use “dry AMD” to refer to the combination of “early AMD” and “GA or late dry AMD disease”, making dry AMD a form that makes up the majority of all AMD cases. In early dry AMD cases, patients suffer several blind spots in the worst scenarios. Only after a series of elusive progression can late Dry AMD (GA AMD) be developed, at which stage patients subsequently entirely lose their central vision [22].

Wet AMD, a more destructive form of advanced AMD, is the result of neovascularization or abnormal angiogenesis in the choroid underneath the macular region. The neovessel formation invades through the Bruch’s membrane and proliferates into the location of RPE and neurosensory retina cells and can lead to several damaging pathological changes [21]. According to the Age-Related Eye Disease Study Research Group definition of the disease, hemorrhage, neurosensory retinal detachment and retinal scar tissue formation are all terms used to reference neovascular AMD [23]. Since the etiology of advanced AMD (wet AMD and GA AMD) remains unclear, scientists have spent considerable effort in finding evidence on the relevance between early dry AMD and wet AMD. Although clinical statistics [24-26] have shown that drusens may increase
the risk of neovascular development, obvious connections between the drusen in early AMD and the neovascularization were unknown until recently. However, recent clinical observations using immunological methods reported that complement components C3 and C5 in drusen direct the expression of Vascular endothelial growth factor (VEGF) and lead to neovascular formation [27]. This suggests that not only is GA AMD a subsequent stage of early dry AMD, but that wet AMD also potentially progresses from the early AMD.

Figure 2 Photographs of healthy eye and eyes with AMD.
This figure is reprinted from [28] Neuron, 75.1, Ambati, Jayakrishna, and Benjamin J. Fowler (2012), Mechanisms of age-related macular degeneration, Pages No. 26-39, Copyright (2017), with permission from Elsevier. (A) normal pigmentation and choroidal capillaries. (B) Geographic atrophy in the eyes with late dry AMD, large drusen formation (yellow deposits) area in the center region. (C) Blood vessel invasion in the Bruch’s membrane and fluid leakage in eyes with wet AMD.

I.3. Treatment of Age-Related Macular Degeneration (AMD) and Drawbacks

Since the pathological features of AMD are diverse, the treatments for early dry AMD, GA AMD and wet AMD are correspondingly different as well. The onset of AMD largely depends on both genetic factors (e.g., the variant of the complement factor H gene [29]) and environmental factors (e.g., smoking, obesity, etc.) and dietary therapy should be paramount towards the
prevention of AMD [30]. But, there is no known cure for wet AMD and there are not even effective treatments for GA AMD [31].

Clinical studies have shown that, for early/intermediate dry AMD, nutritional supplements can have positive effects on slowing the progression to advanced AMD [32-33]. For example, the Age-Related Eye Disease Study (AREDS) [20] used a formulation consisting mainly of anti-oxidant vitamins and minerals. This supplement was found to decrease the risk of progression by 25% over 5 years. Vitamins in the AREDS-type formula usually vary but one example is the use of a combination of vitamin C, vitamin E and beta-carotene, with the role of these aiming to prevent retinal oxidation. Minerals like zinc are also frequently present in the AREDS formulation because the retina utilizes zinc to participate in transporting vitamin A to produce melanin for pigmental protection in eyes [34]. As is reported, if the AREDS formulation were properly prescribed, over 5400 people could be saved from progressing into more dangerous wet AMD [20].

Apart from using AREDS-type formulation, clinical studies have revealed that some other types of nutrients may be beneficial in slowing the progression as well [32]. For example, omega 3 polyunsaturated fatty acids can be useful in preventing the retinal oxidative process and age-related retinal damage [35]. Given the fact that during the development of AMD, the DHA level is reported to be lower than normal [36]. Omega 3 fatty acids, whose structure mainly includes docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), can compensate for the DHA deficiency in the retina. Further, its long chain EPA may also help to postponing retinal oxidation.

Compared to the limited conditions in treating dry AMD, several treatment options are available for wet AMD though, again, none of them provide a cure. In recent decades, treatments focused on the inhibition of VEGF due to its role in wet AMD have become quite popular [37-39]. In human retina, RPE is responsible for both VEGF secretion to activate angiogenesis or vascularization in the retinal choroid and pigment epithelium-derived factor (PEDF) secretion to suppress angiogenesis [40]. Normally, PEDF levels are higher than VEGF levels in retina [41]. However, up-regulated secretion of VEGF as a result of hypoxia or local inflammation in Bruch’s membrane alters the balance. As a response, uncontrolled neovascularization occurs [42]. These uncontrolled new vessels will invade through aged Bruch’s membrane into RPE layers and lead to RPE dysfunction, detachment and cell death [43-45]. Therefore, inhibiting VEGF is a key step in treating wet AMD. In fact, anti-VEGF therapy has already become the most effective and standard
clinical treatment for wet AMD [37, 46]. Anti-VEGF therapy or VEGF inhibition therapy is a method using VEGF inhibitor or monoclonal antibody to prevent choroidal neovascularization. Bevacizumab and Ranibizumab [47] are two such VEGF inhibitors currently available on the market. VEGF-A is known as a chemical signal acting intimately in stimulating angiogenesis, and Bevacizumab is a humanized anti-VEGF monoclonal antibody offering two binding sites to VEGF-A so as to prevent its activity [48]. Compared to Bevacizumab, Ranibizumab is currently the most commonly used inhibitor which has 100 times the VEGF-A binding affinity of Bevacizumab and its price is also less. In double-blind clinical studies, both Bevacizumab and Ranibizumab showed efficacy in vision improvement for wet AMD patients [47-49].

Photodynamic Therapy (PDT) is another popular treatment method for wet AMD, which was developed after Laser Photocoagulation (LP) and soon replaced it. Compared to LP [50], PDT applied a non-thermal lighting and chemical activation method [51] that prevents the high energy stray light from damaging the overlying photoreceptors and causing subsequent irreversible vision loss [52]. In the beginning, PDT was predominantly used in treating acne and it was also reviewed as a feasible technology in treating malignant cancer [53]. Results of PDT clinical trials concluded that it can bring visual acuity benefit to patients with predominant choroidal neovascularization due to wet AMD [54]. In addition, instead of merely using inhibitor injection or PDT alone, an animal study showed that a combination of an intravitreal injection of VEGF inhibitors and a Photodynamic therapy (PDT) with verteporfin can achieve a greater regression in angiographic leakage [55].

Although almost all anti-VEGF treated results in previous studies [48-49, 56-57] state that no significant ocular or systemic side effects have been observed, intravitreal injection procedures do have risks including increased eye pressure, serious eye infection, and even retinal detachment. A recent study on Ophthalmology [58] also indicated that intravitreal injection of inhibitors like Ranibizumab may accelerate GA growth. Further, current anti-VEGF treatment usually requires patients to be injected monthly, and long-term use of intravitreal injections can add risks to patients, especially elderly patients. Compared to intravitreal injection of anti-VEGF, the side effects of PDT are much more prominent. First, PDT cannot restore vision [59-60]. Second, PDT is expensive and has different risk levels for people with different health conditions [61]. One example of the PDT risk is in causing sub-retinal hemorrhage [62]. Third, PDT may not be useful
in all wet AMD cases. As indicated in a study conducted in the United Kingdom, cases of worse visual acuity were reported after giving patients with a mutative form AMD a PDT [63].

I.4. Tissue Engineering Approaches to Correcting AMD

Over the last 30 years, the efficacy of clinical treatments for AMD have improved greatly [46-49, 54-55]. As discussed in the previous section, examples of such clinical treatments include non-thermal Photodynamic Therapy (PDT) and anti-VEGF monoclonal antibody injections. Further, the combination of PDT and anti-VEGF therapies has shown good clinical results [64]. But, a series of corresponding side effects and systemic drawbacks reviewed in the previous section indicate the problems with these therapies. Further, the fact remains that existing treatments are palliative or provide temporary relief, but they are not cures for AMD.

Cell therapy for the treatment of AMD could offer a cure, and such an approach [65] was first introduced in 1997 when a human RPE allograft was successfully transplanted to patients in the sub-macular space. Immunological rejections were later reported, but this demonstrated the feasibility of using RPE transplantation without impeding visual function. More recently, retinal progenitor cells (RPC) [66], induced Pluripotent Stem Cells (iPSC) [67-68] and human embryonic stem cells (hESC) [69-70] have been investigated as the transplantation source as long as proper conditions, growth factors and/or necessary gene manipulations are provided [71]. However, although studies have shown it is feasible to use these cells for therapeutic approaches, prominent challenges must still be overcome before these therapies can be clinically realizable. For example, in neovascular AMD cases, transplantation of normal retinal cells like RPE cannot inhibit overexpression of VEGF. In the case of GA AMD, aged Bruch’s membrane loses the ability to support attachment of RPE monolayers [72], making the utility of the approach questionable. Additionally, the use of hESC for research is still controversial, with the main issue to-date being the destruction of human embryos to provide a source for the cells [73]. Meanwhile, the world’s first clinical trial conducted in Japan with iPSC in retina was suspended due to safety concerns [74]. Although the study was finally resumed in 2016, the regulatory issues associated with clinical use of iPSC in the other parts of the world remain unpromising.

The use of cell-based therapies is one component (i.e., cells) of the tissue engineering triad. The other components of the triad are materials and signals (mechanical or chemical). Tissue
engineering is a versatile technique using the biological and engineering sciences to combine materials, cells and biochemical factors (e.g., growth factors) to replace biological tissue or improve tissue regenerative ability [75]. One example of a key challenge for retinal regeneration is that if cells are delivered alone, the aged Bruch’s membrane is unable to retain an RPE monolayer attachment. As such, tissue-engineered materials that can replace the Bruch’s membrane to promote cell attachment are being investigated for tissue engineering therapies for retinal regeneration [76].

The materials or scaffolds use for tissue engineering are of paramount importance in tissue engineering, and polymeric materials are much more commonly used than metals or ceramics. A key function of polymeric scaffolds in tissue engineering is to provide mechanical support and meeting surface requirements to promote cell attachment and proliferation. A vast number of polymeric materials have been used in tissue engineering applications. Most broadly, these materials can be classified as natural or synthetic polymers [77].

Natural materials usually provide biological and chemical benefits over synthetic polymers. For example, natural materials often possess favorable porosities for cell infiltration and proliferation and most (particularly proteinaceous materials) offer binding sites for cellular functions such as cell binding [78]. One example of natural materials is decellularized matrices, in which all cells are removed from a tissue, leaving the remaining extracellular matrix components as the scaffold material. Using these materials for retina requires a re-cellularization procedure before implantation [79]. Natural polymers like collagen [80], chitosan [81], alginate [82] are also used as scaffolds for tissue engineering. Numerous collagen-based biomaterials have evolved in recent years, they have been used in, for example, engineered bone tissue applications [83-84], lung alveolar scaffolds [85] and artificial skin grafts [86] after polymerization. However, natural materials have some disadvantages in tissue engineering. For example, for the decellularized approach, finding sufficient donor tissue for the decellularization process is a challenge [87]. Moreover, since natural materials are usually soft, their mechanical properties are quite limited in satisfying the diverse conditions of various native tissues or in having tunable properties to match the native tissues [88].

To overcome some of the drawbacks to natural polymers, synthetic polymers are often used in tissue engineering applications. Generally, these materials are biodegradable and can be designed to meet all the required properties for the given application (i.e., various tissues in human
body). In fact, synthetic biodegradable polymeric materials have already been applied to retinal tissue engineering strategies. Examples include poly(caprolactone) (PCL) [89-90], poly(lactic-co-glycolic acid) (PLGA) [91], and poly(methyl methacrylate) (PMMA) [92], which have all been used to fabricate scaffolds. One challenge with synthetic polymers is that they typically do not provide the same level of support to cell attachment, growth, and proliferation as natural materials because they lack cues for cells found in natural polymers such as cell adhesion molecules and integrin binding domains.

Given the benefits of cellular therapies discussed above, the concept of using tissue engineering strategies with cells along with scaffolds and biochemical cues (e.g., growth factors) offers the possibility of AMD cure. Indeed, there are already examples of tissue engineered RPE transplants in animal models. In a rabbit model study [93], a temperature-responsive polymer poly(N-isopropylacrylamide) (PIPAAm) was used to grow cells so as to offer a carrier-free RPE cell sheet, and the results revealed that tissue-engineered RPE transplant have better attachment than cell suspensions.

I.5. Electrospinning towards Retinal Tissue Engineering

Tissue engineered scaffolds are typically characterized by low toxicity, ability to promote cell adhesion, appropriate pore size for cell infiltration, controlled biodegradability, mechanical properties to provide structure support, morphology and function to mimic native ECM, and the ability to promote cell/tissue function [94].

As is mentioned in previous section (I.4), synthetic polymers lack cues for cell attachment, growth and proliferation. One approach to overcome drawbacks associated with synthetic polymers is to make the polymers more biomimetic. For example, in the case of retinal repair, the goal of tissue engineered scaffolds is to mimic the Bruch’s membrane. Thus, the fabrication method should share features of the native Bruch’s membrane. Scientists have found that among five layers of the Bruch’s membrane, fibers are the main structures presented to support cell attachment, especially in the RPE basement membrane layer [5, 7]. The general fabrication methods used to try to mimic tissue architecture include particle leaching [95], phase separation [96], 3D printing [97] and freeze-drying [98]. The rationale for these methods is that they have frequent appearance in porous structures fabrication, similar to many extracellular matrices in body. However, to best mimic the structure of Bruch’s membrane, it is necessary to mimic the
fibrous nature of this ECM of the retina. Electrospinning, a method that is capable of generating fibrous scaffolds, is a particularly appealing fabrication method and it has already been used for retinal engineering [99-101].

For electrospinning, polymers are dissolved in organic solvent. Electrospinning ejects a polymer solution from a syringe needle by applying a high voltage electric field. As the solution travels from the needle to the ground in the circuit, solvents vaporize rapidly due to their volatility and leave only uniform electrospun polymer fibers on the collector, as shown in Figure 3. The diameters of the electrospun nano-fibrous scaffold usually ranges from 3 nm to over 1 µm [102]. Based on this fiber diameter range, it is clear that electrospinning has the capability of generating fibers that mimic those of the native Bruch’s membrane. As discussed in the first section, the thickness of Bruch’s membrane is only about 2-4 µm and studies also measured that the RPE basal lamina is only 150 nm which is equivalent to the thickness of just one or several electrospun fibers.

Fig. 3 The Electrospinning Circuit.
A syringe pump (omitted) expel the viscous polymer solution from the syringe, and Taylor Cone [103] is shaped by liquid surface tension in the electric field. Polymer solution is prepared by mixing polymer with highly volatile solvent. Polymer jet becomes individual fibers during electrospinning where all solvent vaporized.
I.6. Electrospinning of Polycaprolactone (PCL)

Among variety of polymers mentioned in the previous section, polycaprolactone (PCL) is an FDA-approved biomaterial that has already been applied in retinal tissue engineering [89-90, 104]. In a sub-retinal implantation porcine study, electrospun PCL scaffold not only support RPE cell growth and proliferation but also exhibit well toleration in animal body [105].

PCL is a low-cost, synthetic, biodegradable polymer. It is degraded by hydrolysis of its ester bonds into acidic monomers. PCL degradation is generally slower than other FDA-approved polyesters such as PLGA. This would allow it to provide a support for a longer time frame both in vitro and in vivo. For example, in an amphibian Xenopus laevis retinal injury study, it was shown that it took around 30 days for neural retina regeneration from RPE [106]. In a mice in vivo polymer degradation study, PCL took 21 days to reach 46.2% degradation [107], indicating that PCL may degrade on an appropriate time frame for retinal regeneration. Although the time needed for the scaffold to persist in humans after implantation remains unknown, PCL is a good candidate for research in retinal tissue engineering.

One drawback of PCL electrospun scaffolds is their hydrophobicity, which can result in poor cell attachment. However, by modifying the surface of PCL scaffolds in either a physical or a chemical way, cells can better adhere and possibly proliferate. Four surface modification methods have been described for PCL [108]: (1) plasma treatment, (2) chemical etching, (3) protein adsorption and (4) blending with bioactive materials before electrospinning. While, all four methods can be very practical to improve the cell adhesion to PCL scaffolds, protein adsorption is the most straightforward method. A large variety of ECM proteins and other proteins have been shown to promote cell adhesion and can therefore be used in the protein adsorption procedure. These proteins include laminin [109], fibronectin [110], vitronectin [111] and protein combinations like serum proteins [112].

I.7. Keratin Hydrogel

The extracellular matrix of the retina (Bruch’s membrane), as noted above, involves more than just a fibrous matrix. In addition, the retina itself consists of other components such as the
choroid. As such, the mechanical characteristics of the native tissue are complex. In seeking an approach to better mimicking the native tissue, it may be advantageous to have an underlying material below the electrospun mat to play the role of the choroid. The laboratory of my thesis advisor (Dr. Saul) has been investigating keratin biomaterials for this application.

Keratin are proteins that can be derived from many sources including human hair. Based on two different extraction methods, two classes of keratin with different chemical properties can be identified: (1) keratose by oxidative extraction and (2) kerateine by reductive extraction. Previous study [113] shows that kerateine has larger compressive modulus than keratose, and thus kerateine is selected to be the only class of keratin used in this study.

The application of keratin as a novel biomaterial in tissue engineering and regenerative medicine field has gained increasing attention in recent years [114-115]. The shear thinning nature of keratin allows extrusion of hydrogels. Keratin hydrogel is a porous structure with porosity of up to 90% which enables the infiltration of cells as well as vascularization [118]. And similar to natural polymers like collagen, keratin showed minimal immune response [119]. Thus, keratin hydrogel can be promisingly used as a scaffold for vascularization after the implantation. For example, keratin hydrogel act as the scaffolds in nerve [114] and bone regeneration [115-116] and as the carriers in bioactive protein delivery studies [117], exciting progress and results were achieved subsequently. Although keratin as a natural polymer has a limited mechanical performance, studies [113, 120] have shown its good tunability not only in protein release profile but also in mechanical property.

I.8. Electrospun-Aerogel Model for the Study AMD

In the sections above, I have indicated advantages of both electrospun PCL and keratin biomaterials in mimicking aspects of the native retina (e.g., topography and porosity, respectively). One long-term goal of developing these materials is to realize the retinal tissue implantation in patients with AMD. Another goal of developing these materials is for their use in in vitro models to understanding mechanical and chemical effects on cellular processes that might be targeted for therapeutic approaches to treat AMD. As a first step towards these goals, this thesis work seeks to develop a biomimetic retinal scaffold that can support the seeding of RPE cells. This thesis work seeks to characterize electrospun PCL-keratin aerogel scaffolds and to use simple in vitro studies
with the ARPE-19 cell line to investigate the biological utility.

These biomaterial scaffolds combine the electrospun fibers with keratin scaffolds, which are referred to through the remainder of this thesis as a fibrous-aerogel scaffold. Benefits of using this novel combined biomaterial scaffold system includes both features of porous scaffold (mechanical integrity) and fibrous scaffold (ECM-like fibers) that, together, mimic aspects of the native retina. In this thesis, the model is a combination of PCL electrospun fibrous scaffold and porous keratin aerogel, which is a lyophilized (freeze-dried hydrogel) (see Figure 4). This use of an aerogel (rather than a hydrogel) was to prevent “flowing” of the keratin during the electrospinning process as it was noted above that keratin is a shear-thinning material. The use of the aerogel also allowed for longer-term storage of the resulting materials.

The two layers of the combined scaffold serve different purposes. The overlying PCL fibers is to mimic the fiber layers in the Bruch’s membrane to offer morphological similarity to promote cell attachment/adhesion and possibly cell proliferation. The underlying keratin aerogel is to mimic native Bruch’s membrane and choroid complex to provide physical support. It is important to note that the mechanical properties of the Bruch’s membrane change with age [121], and therefore, the tunability of the keratin aerogel mechanical properties is an important aspect of the design.

Figure 4 Native macular anatomy vs. Fibrous-aerogel model system.
The fibrous-keratin aerogel model system seeks to mimic both choroid and partial layers of Bruch’s membrane. The scaffold has two main functions: physically support the overlying structure and implement growth factor delivery to the upper RPE layers after RPE cell seeding. The native macular anatomy on the left is adapted from “The Science of AMD” patient brochure [122].
I.9. Hypothesis and Objective for Thesis Work

The hypothesis guiding this thesis is that the design of PCL/keratin fibrous-aerogel scaffolds can mimic native Bruch’s membrane and facilitate RPE cell functions. Three experimental objectives are identified to test this hypothesis. First, I chose to examine the similarity of the morphology of fibrous-aerogel compared to native Bruch’s membrane. Second, I chose to test the similarity in mechanical properties of fibrous-aerogel with a comparison to literature values for native Bruch’s membrane. Third, I tested the ability of fibrous-aerogel scaffolds in promoting RPE cell attachment and proliferation.
References


CHAPTER II - Fabrication and Characterization of PCL Fibrous-Keratin Aerogels

Ziqian Zeng

This thesis chapter will serve as the basis for a manuscript submission planned to the Journal of Biomedical Materials Research Part A. Major aspects of this chapter may therefore be used in preparation of that manuscript.
II.1. Introduction

Tissue engineering approaches to retinal regeneration offer a potential cure or, at least, an improved treatment strategy for all types of AMD. Moreover, the development of biomimetic scaffolds offers a more complex in vitro system to understand effects on cellular responses to environmental cues such as growth factors or matrix stiffness. Our initial attempt toward the development of such a material to support a tissue engineered retinal implant or model system focuses on development of a fibrous-aerogel model scaffold. This thesis provides the first step to fabricate and characterize this fibrous-aerogel scaffold. Chapter I described the rationale for the material selection. To summarize, polycaprolactone (PCL) and keratin are used as the materials in fabricating the fibrous-aerogel model. PCL [1-4] is an FDA-approved synthetic polymer and keratins [5-9] are naturally-derived materials that have shown low toxicity and good biodegradability in many studies. Previously, PCL was shown to be a suitable scaffold material in retinal implantation [3-4] whereas keratin has been used in various in vivo studies including those for skin, bone and nerve regeneration [5-7].

The fibrous-aerogel design consists of a PCL electrospun fibrous mat on top of a keratin aerogel (see Chapter I-Figure 4). Since the fibrous-aerogel model and the combination of PCL fibers and keratin aerogel have never been shown in literature before, the nano-structure morphology, material mechanical response, as well as the biological response to the material remain unknown. Given our long-term goal of developing materials for retinal regeneration, it is necessary to characterize these materials. Conforming to the criteria [10] discussed previously, I chose to characterize fibrous-aerogel scaffold for morphological similarity to native ECM, compressive modulus, and the biological responses of cell viability and cell proliferation.

Morphological similarity to native ECM provided as a nanofibrous surface in mimicking the Bruch’s membrane in our study allows for the investigation of interactions with cells. Studies have shown that biomaterial fibers are widely-used to mimic collagen fibers in native ECM to help in promoting cellular functions such as providing a reservoir of protein signals (e.g., growth factors) to promote cell growth, proliferation or differentiation [11-12]. In this thesis work, therefore, one study focused on assessing if the fibrous-aerogel could faithfully mimic the native Bruch’s membrane.

Secondly, it is known that the mechanical properties of retina change with age and disease [13-14]. Therefore, the ability to develop a material with tunable of mechanical properties is
another desired feature. In vitro or in vivo, a biomaterial should provide mechanical support to promote cellular proliferation and infiltration; that is, the material literally serves as a “scaffold”. In general, matrix or scaffold stiffness will affect cellular behavior [15]. In addition, biomaterials must at least possess sufficient mechanical integrity to avoid collapse in the presence of physiological forces that would collapse their pore structures [16]. Additionally, in the study of retina and AMD, the elasticity of Bruch’s membrane varies with ages [13] and young people tend to have softer Bruch’s membrane than older people [14]. Therefore, this thesis seeks to test if the fibrous-aerogel could have similar and tunable mechanical property to the native.

Third, the in vitro biological response study provides a way to test the effect of the material on cells. Although the results may be inaccurate in regard to predicting the actual in vivo performance due to differences between the in vitro and in vivo environments, it offers good indications in suggesting whether the material is toxic and can support cell attachment. In this study, the levels of toxicity of the fibrous-aerogel scaffolds on ARPE-19 cells and the effects on cell proliferation were investigated. The effects of the fibrous-aerogel scaffolds on the morphology of attached cells was also investigated by SEM.

II.2. Materials and Methods

II.2.1. Materials

Polycaprolactone (PCL) pellets (Mn=80,000) and methanol (99.8%) were purchased from Sigma Aldrich (St. Louis, MO). Lyophilized keratin powder as α-keratin purchased from KeraNetics, LLC (Winston-Salem, NC), and used without further modification. Chloroform was purchased from Fisher Scientific (Hampton, NH). Cables, alligator clips, electric tapes and PVC pipes were purchased from ACE Hardware Supply (Local Chain Retailer), stainless steel needles and 3 mL syringes were from Grainger Industrial Supply (Lake Forest, IL). Sources of other materials and models of apparatuses were as noted below.

II.2.2. Apparatus Set-up for the Fabrication of Electrospun PCL- Keratin Aerogel Scaffolds

A horizontal electrospinning apparatus (Figure 1) was built for generating the PCL mat of the fibrous-aerogel scaffolds. A syringe pump (KDS Model 100) (Holliston, MA), a high voltage power supply (Glassman High Voltage, PS/EL30R01) (High Bridge, NJ), a ground collector, a
luer-lock polypropylene syringe (3 mL) and a 23rd gauge stainless steel needle were used in creating the circuit. The cable of the ground end was placed inside an inverse “L” shape PVC pipe to enhance the electric isolation. A ground cable was clipped at the end of a zinc screw and the screw head was placed such that it stood out from a plastic electric isolator so that it would offer an area for taping an aluminum foil ground collector. An aluminum foil ground collector was then modified into a square shape whose area is slightly larger than the keratin aerogel so that the cylindrical aerogel could fit directly on to it to support a uniform coating on the scaffold. A large fiberglass cover was placed outside the circuit to provide safety in preventing inadvertent touching of the needle while high voltage was on. Preliminary studies on these modifications to the electrospinning showed that, the time of electrospinning an ~ 100 µm thick fibrous mat could be shortened from 30 minutes for a 2.5cm by 2.5cm size collector plate to about 1 minute by using the methods noted above for our scaffolding system.

II.2.3. Electrospun Scaffold and Aerogel Fabrication

An initial study was conducted to determine the PCL fiber diameters that resulting from electrospinning apparatus under specified conditions so that fibrous-aerogel scaffolds with appropriate fiber diameters could be fabricated. In this study, the electrospinning apparatus was set up as described in the previous section and parameters used in these studies are listed in Table 1. Specifically, the applied voltage was fixed at 19 kV, a syringe pump gave a feed rate of 0.7 ml/h. It should be noted that these values were based on preliminary studies seeking to obtain fibers diameters of ~ 100nm. 5.5%, 6% and 8% (w/v) PCL solutions were made by dissolving PCL pellets in a chloroform/methanol solution (3:1, v/v), followed by vortexing for one day to fully mix and dissolve the PCL. The resulting PCL solutions were loaded in the 3 mL polypropylene syringe minutes before electrospinning. Then, 5%, 6.5% and 8% PCL solutions were electrospun directly on aluminum foil to yield a batch of fibers not associated with keratin aerogel in order to serve as controls. For these electrospun PCL mats, the fibers were peeled off from the foil and stored in a vacuum chamber until used for further experiments.

Separately, lyophilized keratin powders were mixed with deionized water at room temperature to get 7%, 9%, 11%, 13%, and 15% (w/v) concentrations, and then centrifuged with 1500 rpm for 3 minutes. The keratin gels were then packed into a 3mL syringe (no needle)
(Covidien, Dublin) before an overnight incubation to allow gelation. Keratin hydrogels were then frozen at -80°C for 4 hours and freeze-dried by lyophilizer (Labconco Freezone 4.5L system, Kansas City, MO) to give an aerogel. The resulting keratin aerogels were cut into 4 mm lengths (with 8 mm diameter), and taped on same sized aluminum foil collector before electrospinning.

For PCL electrospun fiber – keratin aerogel fabrication, all prepared keratin aerogels were subsequently electrospun with 8% PCL solution at room temperature with the same parameters list in the Table 1. After spinning, a razor blade was used to cut the scaffolds from the foil (which was attached due to the electrospun fibers contacting both the scaffold and the aluminum) and the electrospun keratin aerogel was removed by simply lifting the aerogel part up. All fibrous-aerogels were then stored in a vacuum chamber until used for further experiments. In addition,
**Table 1: Electrospinning Parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syringe Pump Feed Rate</td>
<td>0.7 ml/h</td>
</tr>
<tr>
<td>Applied Voltage</td>
<td>19 kV</td>
</tr>
<tr>
<td>Stainless Steel Needle Gauge</td>
<td>23</td>
</tr>
<tr>
<td>Needle-collector Distance</td>
<td>10 cm</td>
</tr>
<tr>
<td>Aluminum Foil Collector Size</td>
<td>2.5 cm x 2.5 cm</td>
</tr>
<tr>
<td>Polymer Solution for Electrospinning Directly on Aluminum Foil</td>
<td>5%, 6.5% and 8% PCL in Chloroform/Methanol (3:1, v/v)</td>
</tr>
<tr>
<td>Polymer Solution for Electrospinning on Keratin Aerogel</td>
<td>8% PCL in Chloroform/Methanol (3:1, v/v)</td>
</tr>
<tr>
<td>Electrospinning Time</td>
<td>2 minutes</td>
</tr>
</tbody>
</table>

**II.2.4. SEM Morphology Study**

Scanning Electron Microscope (SEM) images were taken to observe the electrospun fibers, the porosity of the keratin aerogel, and the interface between the fibers and the aerogel components of the scaffolds. The following steps were conducted before SEM (Zeiss Supra-35, Germany) imaging: the 7%, 9%, 11%, 13%, and 15% (w/v) keratin with one surface coated by PCL electrospun fibers were mounted on SEM plates and sputter coated (Denton desk II cold sputter unit, NJ) with gold particles for 90 seconds to give an approximately 20 nm conductive gold layer. 500X, 5000X, 20kX, 30kX and 150kX magnification images were then collected with an accelerating voltage of 5 kV.

**II.2.5. Compression Testing**

8% PCL/ 7%, 9%, 11%, 13%, and 15% Keratin fibrous-aerogels (n=6) were taken out from the vacuum chamber before mechanical testing. Dimensions were measured by caliper and subjected
to compression testing on an Instron machine (3344) with a crosshead speed of 10 mm/min. A 100N load cell was used and a preload of 1 N was applied for the testing method. Based on preliminary testing results, material cracks on the fibrous-aerogel were observed to occur at between 30N to 50N loads. Therefore, the test was set to an end when the applied force reached 40N. Force and displacement data were then collected from each individual test, and stress and strain were determined and plotted. Compressive modulus was determined from the linear region of the stress-strain curve.

II.2.6. Biological Response to Fibrous-Aerogel Scaffolds

II.2.6.1. Cell Culture and Cell Counting

ARPE-19 (ATCC® CRL-2302™) cells between passages 6-12 were used for these studies. The cells were removed from frozen storage in liquid nitrogen and warmed at 37°C until thawed. The cells were cultured in filtered (0.2 µm filter) DMEM/F12 (Hyclone™, UT) supplemented with 10% fetal bovine serum (Life Technologies, CA), and 1% Antibiotic-Antimycotic (Invitrogen™, Fisher, MD), which is referred to below as ARPE-19 cell media. Cells were incubated in a 37°C incubator (HERACELL 150i, NC) with 5% CO₂ and ARPE-19 cell media was replaced every other day. Cells were sub-cultured when they reached 80% confluency. At this time, cells were washed twice with 2 mL 0.25% Trypsin (Hyclone™, UT) and then treated with another 2 mL trypsin for 5 minutes in the incubator to allow cell detachment. 8 mL of ARPE-19 cell media was then added to neutralize the trypsin. Cells were centrifuged and resuspended with ARPE-19 and were either sub-cultured or counted if used for experiments. For counting, 100 µL of the cell suspension was mixed with 100 µL 0.4% Trypan blue (Sigma®, MO). 10 µL of this cell-trypan blue mixture were placed on each side of a hemacytometer (Hausser Scientific, PA). Cell numbers were the determined according to the manufacturer’s protocol.

II.2.6.2. Cell Seeding on Fibrous-Aerogel Scaffolds

4 mm height tubular 8% PCL/ 7%, 9%, 11%, 13%, and 15% (w/v) keratin fibrous-aerogel scaffolds were fabricated with aseptic technique as described above and held in place in a 48-well tissue culture polystyrene (Nunclon™ Delta Surface, Denmark) by modified 1.5 mL polypropylene Microcentrifuge tubes (Fisherbrand™, NH) (see Figure 2) to prevent the scaffolds
from floating in cell media due to the high porosity and low density of the scaffolds. All non-sterile materials and tools were autoclaved with the exception that after electrospinning (in non-sterile environment) the resulting scaffolds were subjected to 20 minutes under ultraviolet light to minimize potential contamination during electrospinning process. Fibrous-aerogels were then soaked in PBS (1X) overnight to saturate them, and coated with 200 µL Matrigel (Corning® Matrigel®, NY) for an hour in the 37°C incubator for protein adsorption to the PCL fibers. Free and loosely adsorbed Matrigel were then aspirated out from scaffolds. For cell seeding, 40,000 cells were counted and seeded on each fibrous-aerogel scaffold and cultured with ARPE-19 cell media.

Figure 2. Modified microcentrifuge tube for scaffold fixation in the 48-well plate. Modified 1.5 mL polypropylene MCT tube for preventing fibrous-aerogel floating in liquid media. The height of the modified tube is a little higher than the well. Thus, once the 48-well plate is closed, fibrous-aerogel would be held in place (under the media) in the well by the gravity of the lid.

**II.2.6.3. Cell Viability Testing**

8% PCL/ 7%, 9%, 11%, 13%, and 15% (w/v) fibrous-aerogel scaffolds with ARPE-19 cells as described above were cultured in 48-well plates for 2, 4.5, 7 days. 2 (n=2), 4.5(n=3) and 7 (n=3) days point experiments were conducted initially, additional biological replicate experiments were later conducted at 2 (n=1), 4.5 (n=1) and 7(n=1) days time points. A detailed experimental design is shown in Table 2. At each time point, media was aspirated and scaffolds were treated with LIVE/DEAD™ Viability/Cytotoxicity Kit (for mammalian cells) (ThermoFisher, KY) by the following steps: 5 µL Calcein-AM and 20 µL Ethidium homodimer-1 were mixed with 10 mL
PBS to make the working solution of the reagents. 200 µL was added to each scaffold and then incubated for 30 min at room temperature. The cells were then imaged by epifluorescence microscopy (Nikon Eclipse 80i, Japan) with a 10X objective. Fluorescence filters were set for Calcein-AM (494/517 nm) and ethidium homodimer-1 (528/617 nm) to view live and dead cells, respectively. Additional live cell controls (cells on tissue culture polystyrene) and dead cell controls (rinsed with 70% ethanol for 5 minutes) controls were treated by the same procedure during each day point at the 48-well tissue culture polystyrene. Images were collected on an inverted microscope (Olympus IX51, Japan) equipped with a INFINITY 3 (Lumenera, Canada) camera by using a 10X objective to qualitatively assess ARPE-19 cell viability on fibrous-aerogels.

<table>
<thead>
<tr>
<th>Table.2 Cell Viability Experimental Design</th>
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<td>2 days point</td>
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<tr>
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<tr>
<td>Biological Repeat</td>
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<tr>
<td>4.5 days point</td>
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<tr>
<td>Initial Experiment</td>
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<tr>
<td>Biological Repeat</td>
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<tr>
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<td>Final n=3</td>
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<td>Final n=4</td>
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<td>Final n=4</td>
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**II.2.6.4. Cell Proliferation Study**

Proliferation of ARPE-19 cells on fibrous-keratin aerogels were quantitatively investigated by CellTiter 96 Aqueous Nonradioactive MTS (Promega, Madison, WI). As recommended by the manufacturer, MTS (2 mL) and PMS (100 µL) aliquots were mixed together with 10.5 mL cell media to make the reagent. At 24, 48, 72 and 96 hours culture time, 8% PCL/7%, 9%, 11%, 13%, and 15% (w/v) keratin fibrous-aerogels with ARPE-19 cells (n=3) had the cell culture media removed and were treated with 300 µL reagent and incubated at 37°C for 1.5 hours. Then, 100 µL of solution from each well was transferred to a 96-well plate. A microplate reader (Biotek Synergy HT) was used to read the absorbance at 490 nm.

To generate a standard curve for MTS absorbance (at 490 nm) as a function of the number of cells, known numbers of cells were placed on 48-well tissue culture polystyrene. 1x10^5, 5x10^4, 2.5x10^4, 1.25x10^4, or 6250 ARPE-19 cells were seeded in the plate (n=1) with 500 µL media and incubated at 37°C for 6 hours. Cells were then incubated with MTS/PMS as was done for the
scaffolds before the absorbance reading at 490 nm. An absorbance (490 nm) versus cell number (as determined by original plating number) calibration curve was then generated.

In the biological replicate experiment, the experiment with cells on scaffolds was repeated as described above with the modification that 10,000 cells were seeded per scaffold (n=3) in order to insure that the observed results were not due to any saturation in the MTS absorbance reading for the 40,000 cells per scaffold experiment. In this experiment, the calibration curve was also repeated as described above with the exception that the number of cells was 5 x 10^4, 2.5 x 10^4, 1.25 x 10^4, 6250, 3125, 1563, or 0 ARPE-19 cells. To address the seeding variability, this calibration curve was prepared in triplicate (n = 3).

II.2.6.5. Morphology of ARPE-19 Cells On Fibrous-Keratin Aerogel determined by SEM

The morphology of ARPE-19 cells on fibrous-keratin aerogel scaffolds was further investigated by SEM. The cell-scaffolds samples were from 8% PCL/ 7%, 9%, 11%, 13% and 15% (w/v) keratin fibrous-aerogels (n=3) at a 4.5 days point. Equal numbers of cells were seeded on glass coverslips as controls. All cell samples were fixed in 10% neutral buffered formalin for 2 hours and soaked in hexamethyldisilazane (HDMS) for dehydration. Samples were then sputter-coated for 90 seconds to allow a 20nm gold coat. For SEM, low (600X) and high magnification (6000X and 20 kX) images were collected to observe and compare cell morphology and cell interaction with the fibrous-keratin aerogels.

II.2.7. Statistical Analysis

All quantitative experimental results including compressive modulus results and MTS assay absorbance reading results in this study, were subjected to a single-factor analysis of variance (ANOVA) followed by a post-hoc Tukey’s honestly significant difference (HSD) analysis for equal sample size or a Tukey-Kramer analysis for unequal sample size. P values less than 0.05 between groups were taken to be statistically significant.
II.3. Results

II.3.1. SEM Morphology of Scaffolds

To observe the morphology the scaffolds and to observe if the PCL and keratin were in the distinct locations that were part of the original design, SEM images (n=3) of fibrous-keratin aerogels were taken at 500X magnification to observe the PCL fiber, keratin pore, and interface regions, as shown in Figures 3, 4, and 5, respectively. Additional 5000X magnification images were taken on fibers to better observe the fibrous morphology (inserts in Figures 3, 4, and 5). Figures 3, 4, 5 show comparison of PCL fibers, porous structure and interfaces on 8% PCL/ 7%, 9%, 11% ,13% and 15% (w/v) keratin fibrous-keratin aerogels. Separately, native ECM fibers photos from newt Bruch’s membrane were taken by a graduate student in the group of several collaborators (Lam Phuong advised by Drs. Robinson and Del-Rio Tsonis in the Department of Biology and Miami University). Representative images of 8%, 6.5%, 5% electrospun PCL fibers (n=3) only (no scaffolds and images at 30kX magnification and native ECM fibers at 150kX are shown in Figures 6 and 7, respectively.

Empirically, the morphology of fibers, pores and interfaces were very similar among the five experimental groups of different keratin weight percent. In Figure 3, from the inset 5000X magnification fiber photos, the diameters of fibers were found to be similar. In 500X photos, fibers were randomly aligned and tended to have thinner fibers on pores of the keratin. I did not observe large gaps or holes in the fibers or at the fiber-aerogel interface, providing an indication of good adhesion between the electrospun fibers and the underlying keratin aerogel. Figure 4 shows that the keratin parts of the scaffolds had expected pore structures associated with aerogels [17]. Qualitatively it appears that 7% keratin aerogels had the largest pore size among all groups and there is a slight decrease in pore size between 7% to 13%. As can be seen by the cross-section images (Figure 5), scaffolds were revealed to have only a thin layer of PCL fibers over the keratin aerogel pores, as I had intended in order to mimic the thin Bruch’s membrane.

In Figure 6, despite the magnification difference in PCL fiber images (30kX) and ECM fibers in the newt Bruch’s membrane image (150kX), the morphology of PCL fibers are observed to be similar to the newt ECM fibers. In Figure 7, both newt Bruch’s membrane image and PCL fiber images are at 150kX, measurements of fiber diameter are shown in each image. By randomly measuring 90 fibers in three samples at each PCL concentration, the 8% PCL fibers have the average largest diameter of around 700 nm, fibers in 6.5%, 5% PCL photos are around 170 nm.
and 110 nm while the ECM fibers in newt appears to be around 60 nm. This result indicates that by lowering the concentration of PCL, the fiber diameter can be decreased dramatically from ~700 nm (8% PCL) to ~100 nm (5% PCL). And it is very possible that < 50 nm ECM fibers can be achieved by further decreasing PCL concentration. Additionally, beads formations were observed in 6.5% and 5% PCL samples.

Figure 3. Comparison of fibrous-aerogel PCL fiber region for samples with varying keratin concentrations. Magnifications is at 500X (inset is 5000X magnification). White scale bar represents 100 µm and red scale bar (inset) represents 5 µm. Concentrations of keratin aerogels are (a) 7%, (b) 9%, (c) 11%, (d) 13%, and (e) 15% for each photo. PCL deposited as a electrospun layer on top of each keratin aerogel, and the topography of fibrous scaffold generally resembled each other.
Figure 4. Comparison of fibrous-aerogel porous keratin aerogel region for scaffolds with varying keratin concentrations. Magnification is at 500X and scale bar represents 100 µm. Concentrations of keratin aerogels are (a) 7%, (b) 9%, (c) 11%, (d) 13%, and (e) 15%.

Figure 5. Comparison of fibrous-aerogel fiber-pore interfaces for scaffolds of varying keratin concentrations. Magnification is at 500X and scale bar represents 100 µm. Concentrations of keratin aerogels are (a) 7%, (b) 9%, (c) 11%, (d) 13%, and (e) 15%.
Figure 6. Comparison of 8%, 6.5%, 5% PCL fibers (30kX) and ECM fibers in newt Bruch’s membrane (150kX). (a) 8% PCL, (b) 6.5% PCL, (c) 5% PCL, and (d) ECM fibers in newt Bruch’s membrane. Beads formation were observed in (b) and (c), and 5% PCL tended to have more beads. Scale bars in (a), (b) and (c) were the same and represent 5000 nm and scale bar in (d) represents 200 nm.
II.3.2. Compression Testing

Compression tests were conducted on 8% PCL/7%, 9%, 11%, 13% and 15% (w/v) keratin fibrous-aerogels (FA). Only scaffolds that were not observed to have any defects in their shape or structure after cutting were selected for compressive testing, which resulted in 4 or 5 scaffolds for compression testing. Compressive moduli of fibrous-aerogels were determined from the linear region of the stress-strain curve, and 5% to 15% strain regions were used in calculations based on the fact that this was in the linear region for every tested scaffold before compressive failure was observed (cracking of the scaffolds under compression).
Figure 8 shows the compressive modulus comparison of fibrous-aerogel scaffolds among the five groups of varying keratin concentration. Moduli were found to be higher in scaffolds with greater amounts of keratin. Among the five groups, 7% keratin fibrous-aerogel scaffolds had the lowest compressive modulus while 15% keratin fibrous-aerogel scaffolds had the highest compressive modulus. The moduli ranged from 1.32 MPa to 8.33 MPa.

ANOVA followed by the Tukey-Kramer post-hoc analysis (for unequal sample size) indicated that the moduli of all five groups were significantly different from each other, except for the modulus of the 11% and 13% fibrous-aerogel scaffolds were not statistically different.

Figure 8. Compressive modulus average with error bar (standard deviation). @, #, $, %, & denote values that differ significantly from 7%, 9%, 11%, 13%, 15% keratin fibrous-aerogel (FA) respectively.
II.3.3. **Live/Dead Assay**

To investigate cell viability, toxicity, and morphology, I conducted a live/dead imaging study on ARPE-19 cells cultured on the scaffolds for 2, 4.5 or 7 days. Live/dead images were taken at 10x magnification on fibrous-aerogel scaffolds with 7%, 9%, 11%, 13% and 15% (w/v) keratin concentrations. Figures 9, 10, 11 show representative images of green and red channels for live and dead staining, respectively, on the five groups of fibrous-keratin aerogel scaffolds or on cells cultured on tissue culture polystyrene (controls). The initial 2 day time point images (n=2) results matched those of the biological repeated experiment (n=1), and there were no noticeable differences in the biological replicates. As such, the images shown are representative. Although some of the scaffolds lacked dense areas of live cells (see Figure 9 a1, b1, and e1 for 7, 9, and 15% keratin), dense areas were seen in the 11% and 13% keratin (Figure 9, c1 and d1, respectively). However, at 4.5 (n=3) and 7 (n=3) day time points, there were dense areas of live cells for each keratin concentration (Figures 10 and 11, respectively, and sub-images a1 – e1 for the live cells). In particular, live images (a1 – e1 in Figures 10 and 11) revealed cell monolayer formation.

The three-dimensional nature of the scaffolds meant that it was not possible to have the entire field of view in the same focal plane. This can be seen in Figures 9 – 11, where some regions are in focus while other regions are not in focus. Therefore, a “panorama” image was prepared in which a single field of view was imaged in several focal planes and the parts of the focal plane that were in focus for each image were compiled together to show the nature of the monolayer and cell morphology. Figures 12 and 13 show one representative monolayer formation region for each keratin group.

Many fewer cells were observed in dead (red) images compared to the live (green) images (Figures 9, 10, 11). There were almost no dead cells for all five groups at 2 days point and 4.5 days point. However, the observed numbers of dead cells increased at 7 days and small regions of dead cells were observed to be already detached from the scaffold surface.
Figure 9. Fibrous-aerogel scaffold live/dead fluorescent microscopy images at 2 day time point on the scaffolds (8% PCL) with different concentration keratin. Keratin concentrations are (a) 7%, (b) 9%, (c) 11%, (d) 13%, (e) 15%. Tissue Culture Polystyrene controls are, (f) live cell control on TCPS, (g) dead cell control (treated with ethanol) on TCPS; For all fluorescent photos, 1- green channel for live cell staining, 2- red channel for dead cell staining.
Figure 10. Fibrous-aerogel live/dead fluorescent microscopy images at 4.5 day time point on the scaffolds (8% PCL) with different concentration keratin. Keratin concentrations are (a) 7%, (b) 9%, (c) 11%, (d) 13%, (e) 15%. Tissue Culture Polystyrene controls are, (f) live cell control on TCPS, (g) dead cell control (treated with ethanol) on TCPS; For all fluorescent photos, 1- green channel for live cell staining, 2- red channel for dead cell staining.
Figure 11. Fibrous-aerogel live/dead fluorescent microscopy at 7 day time point on the scaffolds (8% PCL) with different concentration keratin. Keratin concentrations are (a) 7%, (b) 9%, (c) 11%, (d) 13%, (e) 15%. Tissue Culture Polystyrene controls are, (f) live cell control on TCPS, (g) dead cell control (treated with ethanol) on TCPS; For all fluorescent photos, 1 - green channel for live cell staining, 2- red channel for dead cell staining.
Figure 12. Panoramic cell monolayer live/dead images at 4.5 days point. Panoramic images show cell monolayers at 4.5 days on the scaffolds (8% PCL) with different concentration keratin and keratin concentrations are (a) 7%, (b) 9%, (c) 11%, (d) 13%, (e) 15%. For all fluorescent photos, 1- green channel for live cell staining, 2- red channel for dead cell staining.

Figure 13. Panoramic cell monolayer live/dead images at 7 day time point. Panoramic images show cell monolayers after 7 days on the scaffolds (8% PCL) with different concentration keratin, and keratin concentrations are (a) 7%, (b) 9%, (c) 11%, (d) 13%, (e) 15%. For all fluorescent photos, 1- green channel for live cell staining, 2- red channel for dead cell staining.
II.3.4. MTS Assay

To provide a quantification of the cell viability values and to investigate proliferation of the cells, I used an MTS assay. In the first experiment, 40,000 cells were seeded per scaffold and absorbance readings (490 nm) on the five groups (n=3) were taken by microplate reader at 2, 4, 6 and 8 day time points. Figure 14 shows the calibration curve of cell number versus absorbance at 490 nm and Figure 15 shows ARPE-19 proliferation with time based on the standard curve of Figure 14.

![Figure 14. Cell number vs. absorbance (at 490 nm) calibration curve (First Experiment). This calibration curve is prepared with n=1.](image-url)

Cell Number = 6.82x10^4 * (A_{490}) - 1.19x10^4
R^2 = 0.909
Figure 15. Cell number vs. time (First Experiment). 40,000 cells per scaffold seeding density for the 5 different keratin concentrations (n=3). Error bars represent standard deviation.
Single factor ANOVA analysis with Tukey’s HSD post hoc test (for equal sample size, n=3) were conducted on cell numbers at 2, 4, 6, 8 day points for comparison between samples at each time point and within each sample at the various time points. Results of this statistical analysis are shown in Table 3 (comparison of each sample at different times) and Table 4 (comparison between formulations).

### Table 3. Single factor ANOVA with Tukey’s HSD analysis on cell number of different day points (First Experiment)

<table>
<thead>
<tr>
<th></th>
<th>7% keratin</th>
<th>9% keratin</th>
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“-” means no significant difference, “+” means significant difference

### Table 4. Single factor ANOVA with Tukey’s HSD analysis on cell number of different keratin concentration fibrous-aerogel (First Experiment)

<table>
<thead>
<tr>
<th></th>
<th>7% vs. 9%</th>
<th>7% vs. 11%</th>
<th>7% vs. 13%</th>
<th>7% vs. 15%</th>
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“-” means no significant difference, “+” means significant difference
In the second experiment (experiment to show repeatability), 10,000 cells were seeded on each scaffold to ensure that the MTS signal was not saturated and that there was physical area on the scaffold over which cells could proliferate. The absorbance readings (490 nm) on the five groups (n=3) were taken by microplate reader at 2, 4, 6 and 8 day time points. Figure 16 shows calibration curve of cell amount versus absorbance at 490 nm and Figure 17 shows ARPE-19 cell amount changing in time. To address the variability in cell seeding, the calibration curve was prepared in triplicate (n = 3).

\[
\text{Cell Number} = 4.50 \times 10^4 \times (A_{490}) - 9.69 \times 10^3
\]
\[ R^2 = 0.95 \]

Figure 16. Cell number vs. absorbance (at 490 nm) calibration curve (Second Experiment). This calibration curve is prepared with n=3. Error bars represent standard deviation of absorbance readings.
Figure 17. Cell number vs. time (Second Experiment). 10,000 cells per scaffold seeding density for the 5 different keratin concentrations (n=3). Error bars represent standard deviation.
Single factor ANOVA analysis with Tukey’s HSD post hoc test (for equal sample size, n=3) were conducted on cell amount at 2, 4, 6, 8 day points of each individual keratin concentration. And then, single factor ANOVA analysis with Tukey’s HSD post hoc test were conducted on cell amount of different concentrations at each individual time points. The results of these analyses are shown in Tables 5 and 6.

<table>
<thead>
<tr>
<th>Table 5. Single factor ANOVA with Tukey’s HSD analysis on cell number of different day points (Second Experiment)</th>
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<tr>
<td>7% keratin</td>
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“-” means no significant difference, “+” means significant difference

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<th>Table 6. Single factor ANOVA with Tukey’s HSD analysis on cell number of different keratin concentration fibrous-aerogel (Second Experiment)</th>
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“-” means no significant difference, “+” means significant difference
II.3.5. Morphology of ARPE-19 Cells on Fibrous-Aerogel

After conducting the live-dead experiments, I fixed the cells on the fibrous-aerogel scaffolds in order to investigate their morphology and appearance, particularly at higher magnifications, by SEM. Comparisons of cells on the different keratin concentration fibrous-aerogel scaffolds (n=3) and cells on coverslips (controls, n=3) were imaged at 500X magnification (Figure 18) to observe the general cell distribution on the scaffolds. 6000X magnification was used to investigate individual cell morphology and inset photos of even higher magnifications (at 20kX) were taken to observe cell interactions with fibers of the fibrous-aerogel scaffolds (Figure 19).

Figure 18. SEM micrographs of ARPE-19 cells on fibrous-aerogel at low magnification (500X). Micrographs show that the ARPE-19 cells grown on the surface of different keratin concentration after 4.5 days in culture. No ARPE-19 grew through (or underneath) the PCL fibers into the keratin region of the scaffold. (a) ARPE-19 on glass cover slip, (b), (c), (d), and (e) are ARPE-19 grown on 7%, 9%, 11%, 13% and 15% keratin, respectively. Note that 7% (b) and 11% (d) keratin scaffolds show similar monolayer formation and cell morphology to cells on TCPS (a). Scale bar of 100 µm is shown in (a).
Figure 19. SEM micrograph of ARPE-19 cells on fibrous-aerogel at high magnification (6000X and 20 kX). SEM micrographs of ARPE-19 cells at high magnification (6,000X) on the surface of glass (a) or different keratin concentration scaffolds after 4.5 days in culture. (a) is ARPE-19 on coverslip, (b), (c), (d), (e) and (f) are ARPE19 grown on 7%, 9%, 11%, 13% and 15% PCL, respectively. The red squares show the location of the even higher magnification region. 20 kX magnification images in (b), (c), (d), (e) and (f) show that fibrils of individual ARPE-19 cell are intertwined with PCL fibers. Scale bar of 5µm (6,000X) is shown in (a). Scale bar of 200 nm (20kX) is shown at inset image of (a).
References


Chapter III - Discussion and Future Directions

Ziqian Zeng
III.1 Discussion

III.1.1 Rationale Discussion on Characterization Methods

A functional Bruch’s membrane, whose functions include physically supporting RPE cells, promoting RPE cell growth and proliferation, regulating nutrient and waste diffusion, and forming a barrier to prevent choroid migration [1], is pivotal in supporting a healthy and fully functional retina. Tissue engineering scaffolds provide an opportunity to better understand the mechanisms of injury and repair in an *in vitro* system and could ultimately be used for therapeutic approaches to treat Macular Degeneration. To serve as an *in vitro* model system, the design for tissue engineered scaffolding systems should allow modulation of these functional characteristics within physiologically-relevant parameters. Towards this end, design criteria for a tissue engineered scaffold might include the ability to manipulate the morphology of ECM-like membrane, the mechanical properties of the scaffolds, and drug release from the scaffolds, among others. Such systems may allow better understanding of these conditions on cell attachment, growth, proliferation, migration, and even differentiation. The rationale behind these criteria and how they might be applied in an AMD *in vitro* model or a further tissue engineered retinal transplant are as follows.

First, the goal of tissue engineering is to replace or regenerate living tissues to as substitutes for injured or diseased tissue. A cell-scaffold substrate that promotes native tissue structure and function would obviously achieve this goal [2]. Thus, scaffolds with the capability of promoting appropriate cellular functions is essential.

Secondly, native Bruch’s membrane is a five-layer structure that consists mainly fibers of ECM components [3]. As such, the morphological similarity of a retinal scaffolding system is needed in order to fully mimic the RPE basal lamina in achieving the native RPE cell morphology. Although cells can be grown in a 2-D environment, to more fully capture their native state it is necessary to provide a three-dimensional system to better promote cellular differentiation and migration [4]. According to a study by Warnke, et al. [5], human RPE behave differently on 3-D nanofiber scaffolds like collagen and PLGA electrospun fibrous sheets than on flat surfaces like PLGA film and cover glass. Although monolayers were observed on all four types of seeding surfaces, hexagonal RPE that resembled native human RPE morphology were only found on electrospun fibrous sheets. Moreover, a fibrous scaffolding system can be beneficial in the delivery
of nutrients and oxygen after clinical implantation because its porosity provides a permeable system for small diffusion [3].

Thirdly, ECM stiffness is known to have effects on cell behavior [6-8]. According to these studies, the mechanical performance of a local matrix is intimately related to cytoskeletal organization which may influence cellular development and differentiation. Specifically, a relatively stiffer ECM can retain a more confluent cell layer and can promote more rapid cell proliferation and differentiation. One example where the variability in tissue elasticity [9] can be observed to have a function effect is in the effects of the Bruch’s membrane mechanical properties in the aging process [10]. For this reason, a manipulation of mechanical properties in the fibrous-aerogel scaffolding system in this thesis would allow for the effects of matrix stiffness to be studied on outcomes such as RPE cell proliferation and differentiation. In literature [7-9], many properties including shear modulus, compressive modulus, tensile strength are measured to offer a complete image of ECM mechanical performance. In this thesis, the compressive modulus of dehydrated PCL/keratin fibrous-aerogel was compared to that of dehydrated Bruch’s membrane and choroid complex compressive modulus reported in the literature [10].

Finally, drug delivery ability in associate with diffusivity of the scaffolding system may be another important factor I would like to have as this would allow an understanding of the temporal effects of certain endogenous or exogenous agents (i.e., drugs) on retinal tissue formation. Bruch’s membrane is a porous structure that regulates material transportation in retina. All nutrients, oxygen, signaling molecules, and metabolic waste passively diffuse through the Bruch’s membrane to keep a dynamic balance in the retina [3]. Diffusivity of Bruch’s membrane is believed to be controlled by its elasticity, and failure in diffusion control by the loss of elasticity may play important role in AMD development [10]. Thus, the ability to tune the mechanical properties and, subsequently the degradation or drug delivery properties of the scaffold may allow for the study of metabolic mechanisms in the retina and the pathology of disease development in an in vitro system.

III.1.2 Summary of Thesis Findings and Interpretation of Results

As described above, the mechanical properties of the cell substrate play an important role in cell behavior and a tissue-like stiffness has effects on directing cell differentiation [11]. In
addition, the mechanical properties of human Bruch’s membrane change with age [10]. For these reasons, a key aspect in the design of scaffolds for models of retinal regeneration are tunability in the mechanical and morphological features of the materials. Based on SEM images of the scaffolds and compression testing data analysis, the PCL-keratin fibrous-aerogel model showed tunability in the morphology of the fibers for mimicking the Bruch’s membrane (~700 nm to less than 150 nm fibers) and also tunability of the compressive modulus (1 MPa to larger than 8 MPa) in achieving similar range of native Bruch’s membrane stiffness (3 MPa- 19 MPa) [10]. As such, the scaffold meets with the design criteria of having tunable morphological and mechanical properties to allow for the use of this system in modeling aspects of retinal injury and regeneration. In addition, this allows for future optimization of the scaffolding system to best achieve regeneration should these materials be used for in vivo models of regeneration and repair of AMD or other diseases/injuries of the retina.

Another key design criteria are that the materials must also be suitable for use with cells and, ultimately, compatible with biological implantation if used for in vivo repair strategies. The initial experiments in this regard focused on live-dead and cell proliferation assays with ARPE-19 cells (an immortalized human RPE cell line) to determine whether these criteria were satisfied for PCL fibrous-keratin aerogel scaffolds. Live/dead images showed large numbers of live ARPE-19 cells (green) and very few dead cells (red). Meanwhile, results of the MTS assay also indicated plenty of viable cells on the scaffolds. Thus, these results offer initial evidence that the PCL-keratin fibrous-aerogel scaffolds have low toxicity to ARPE-19 in cell media.

Another important observation from the live-dead assays that was confirmed by SEM images was the morphology of the ARPE-19 cells on the scaffolds. Both of these studies showed that ARPE-19 cells formed intact monolayers or near monolayers on top of the fibrous-keratin aerogel scaffolds. This kind cell behavior corresponds to RPE monolayers in native retina and indicates that, at least for the ARPE-19 cell line, the cellular response to the fibrous-keratin aerogel is favorable. In the native retina, monolayer formation is essential in facilitating RPE function. For example, an RPE monolayer interact apically with rod/cones at the outermost layer of photoreceptors and this polarized organization of RPE is crucial to the function of neural retina in facilitating vision [12]. According to one study [13], the organization of a polarized RPE monolayer is less likely to suffer immune rejection compared to non-polarized RPE structural organization. In terms of a clinical tissue engineered approach, an RPE monolayer on scaffolds
would be an easier way to handle during the transplantation surgery [14]. It is also noteworthy that the ability to achieve a monolayer on these fibrous-aerogel scaffolds suggests that other cell types can be tested to determine if they show a similar behavior. For example, it would be possible to move to primary RPE including those from various species or those derived from stem cells (including human).

An important aspect of these experiments was the scaffold preparation and cell seeding protocol. In preliminary studies (not shown in this thesis), ARPE-19 cells that were added directly to the scaffolds had low levels of attachment and had poor morphology in that they were rounded and did not look the same as ARPE-19 cells on TCPS. I therefore investigated the use of a protein adsorption strategy to achieve a scaffold surface more favorable for cell attachment. In these studies, scaffolds were pre-coated with Matrigel, which was subsequently removed after one hour incubation. Because the Matrigel was added and incubated for one hour at 37°C, one consideration was the possibility that the Matrigel had formed a layer on top of scaffolds and that the cells actually bound to the Matrigel instead of the PCL fibers [15]. However, in the after-cell-seeding SEM images indicated no noticeable amount of Matrigel on the surface of the scaffolds, which I interpret to indicate that proteins from the Matrigel had adsorbed to the PCL fibers to promote cell attachment. Under these conditions, a gel would be expected, so I suspect that the gels were weak and came off upon aspiration. Again, though, the SEM images revealed no indication of residual gel material. Further, the cellular interactions appeared to be with individual PCL fibers (Chapter II, Figure 15), highlight the importance of both the fibers and the adsorbed Matrigel proteins.

I did not see evidence, in the live/dead or SEM images, of cells penetrating the electrospun layer and moving into the keratin aerogel pores consistent with other reports on electrospun materials. Indeed, it is well-known that achieving cell penetration into electrospun sheet is difficult unless the pores are enlarged [16]. Several other studies also indicate that electrospun sheets are able to retain cells and prevent cell penetration [17-18]. However, SEM images from the keratin aerogel region SEM showed large pores that could be used for cell infiltration in the future. This is an important consideration because the RPE monolayer on the Bruch’s membrane is only a single factor in the overall structure of the retina. Another component of the native retina that might be considered in the future is the vascular structure, which is critical to retinal function [19]. A study by Kraehenbuehl, et al [20] stated their success in seeding vascular cells into a hydrogel. Thus, it seems that it would be possible to have vascular cells infiltrate the porous keratin region
of the fibrous-aerogel scaffolds. This could be beneficial in an in vivo system to promote cell viability to maintain function. However, this approach could also be beneficial in an in vitro model of retinal disease given the fact that vascular cell infiltration is a fundamental component of the etiology of AMD. Strategies would be required to achieve vascularization of the keratin region of the scaffolds. One example would be the use of drug delivery strategies to promote or study the effects of growth factors on vascular structure formation. For example, the delivery of growth factors such as VEGF and PEDF [3] could be used to promote survival and network formation of endothelial cells.

Although I have seen large cell viability and confluency on the fibrous-aerogel scaffolding system, the custom-designed cell seeding protocol for fibrous-aerogel may create experimental variability. The cell seeding was achieved by pipetting 100 μL of a known concentration cell suspension onto the scaffolds (after Matrigel coating). Due to the uneven fibrous-aerogel surface, cell suspension placed at the center of the surface may flow away from the seeding location. This may lead to differences in cell seeding densities or the distribution of cells on the surface. This may account for less monolayer formations at 7 days in some scaffolds in the live/dead experiment. This demonstrates that a careful cell seeding is necessary to achieve higher confluency of cells for all groups of samples.

The MTS assay allowed for determination as to whether cells can proliferate on the PCL/keratin fibrous-aerogel scaffolds. ANOVA and Tukey’s HSD analysis showed that very few significant differences in cell numbers between groups or time points in both experiments (40,000 cells per scaffold or 10,000 cells per scaffold seeding). Stated differently, the same number of cells were present on different keratin concentrations scaffolds at 2, 4, 6, 8 days points, indicating minimal proliferation. These results do not necessarily mean that the scaffold is toxic to ARPE-19 cells since monolayers were observed at 4.5 and 7 days on both live/dead assay results and SEM results. The result that monolayers were observed at 4.5 and 7 days, but not at 2 days on the live-dead assay would seem to suggest that cell proliferation has occurred. However, the MTS result does not indicate that cell proliferation occurred. One possible reason for this observation is that proliferation of ARPE-19 cells on scaffolds with Matrigel adsorption is very slow. Another possibility is that the current protocol of MTS assay cannot get the correct reading of absorbance as the keratin may absorb some of the MTS assay product (formazan derivative). The keratin aerogel is a freeze-dried scaffold which acts like a sponge in cell media, the current MTS assay
protocol is to add 300 µL MTS reagents on the scaffolds right after taking away cell media. It is interesting to note, however, that the number of cells observed are in the same range (same order of magnitude) as the number of cells originally seeded, indicating that the absorbance values are reasonable.

In considering the results of the live/dead and MTS assays, it is troubling that the results seem disparate. The MTS assay results show very little (if any) statistically significant ARPE-19 cell proliferation. But for the live/dead assay, it is clear that more cells are present at later time points (see Chapter II, Figure 9-13), which would require cell proliferation. The live/dead results are confirmed by the ARPE-19 morphology SEM results (see Chapter II, Figure 18-19). Thus, it seems likely that proliferation of ARPE-19 cells occurred on the scaffold. To reconcile these results, the MTS assay protocol may be refined or other methods such as Bromodeoxyuridine (BrdU) labeling might be used in the future to investigate cell proliferation on the scaffolds to resolve this complex result.

III.2 Future Directions

III.2.1 Improving Electrospinning and Fiber Deposition for Fibrous-Aerogel Fabrication

Fiber diameter was an important aspect of the fibrous-aerogel morphological design and characterization. In particular, it was found that native Bruch’s membrane has fiber diameters of approximately 60 nm [5, 21] and Chapter II, Figure 6d. To create biomimetic scaffold, it is necessary to approximate this diameter as closely as possible or to be able to tune the fiber diameters to study and optimize cell attachment, growth, migration and/or differentiation processes as needed.

Studies have shown that parameters of the electrospinning apparatus are important in tuning diameter, alignment, and morphology of fibers [22]. Important parameters include viscosity of the polymer solution, needle gauge, polymer feed rate, applied voltage and size of ground collector. I conducted a preliminary study on electrospinning parameters and found that, for this system, viscosity of the polymer solution was the main parameter that could significantly tune the fiber diameter. Although this system was able to quickly coat the keratin aerogel (i.e., it takes only a few minutes to cover a 4 mm-diameter-aerogel), drawbacks of this design were also found. One example of this is that during the electrospinning process, although I had already created a good
electric isolation around the circuit, the electrospinning jet was not well-controlled. I used a horizontal electrospinning apparatus set-up. Due to gravity effects, the electrospinning jet was also observed to have some vertical movement. There was also some fluctuation of the applied voltage due to power supply variability, and it was therefore challenging to get a uniform fiber sheet, especially when electrospinning very thin (< 200 nm) fibers. Moving forward, a vertical apparatus set-up could provide a solution to these problems.

The viscosity of the PCL solution as the main tunable parameters created a dilemma when trying to achieve extra thin homogeneous fibers by electrospinning. From fibers SEM results among 8%, 6.5% and 5% PCL (see Chapter II Figure 6), formations of beads were observed in the latter two concentrations. A previous study on electrospinning with different PCL concentration showed similar results that beads were formed in less viscous PCL solutions [23]. Possible explanations for the bead formation is the incomplete filament stretch during electrospinning due to large polymeric surface tension [24], low viscosity of the polymer solution [25] or low charge density [26]. Therefore, elimination of fiber beads may require adding surfactants in the solution to lower polymeric surface tension or increase polymeric viscosity or increasing net charge density [26]. As such, the best way to eliminate beads may be to apply a higher and steadier voltage on low concentration PCL solution.

In a review of electrospinning literature [27], it has been suggested that fiber diameter can be tuned under 100 nm by blends of PCL and gelatin or PCL and collagen with less beads because gelatin and collagen are electroactive polymers that had helped increase solution net charge density. In fact, in a preliminary study (not included in this thesis work) our group had sought to adopt PCL/gelatin solutions as the source material for the electrospun fiber mats. However, the experimental results showed that PCL as a hydrophobic material could not be well-mixed with the hydrophilic gelatin using simple solvents combinations (chloroform/methanol and acetic acid in the study) to make a miscible solution. Although several groups have reported success with this solvent combination, most groups report that tetrafluoropropanol (TFP) [28] or hexafluoropropylene (HFP) [29-30] are more suitable solvents for dissolving both gelatin or collagen and PCL together. In the future, the use of gelatin may be beneficial as this protein promotes cell binding without further surface modifications. That is, one possible future direction of my project would be using HFP or TFP based PCL/gelatin solution to fabricate electrospun sheet.
III.2.2 Self-Assembling Materials and 3D Printing as Alternative Scaffolding Systems

To-date, the electrospinning technique has been working well in fabricating larger diameter continuous nanofibers for the fibrous-aerogel scaffolds. But the bead formation at a smaller fiber diameter indeed made the scaffold topography less than ideal in mimicking the native Bruch’s membrane. As noted, the native Bruch’s membrane is near 60 nm, and it was found that 5.5% PCL could achieve ~ 100 nm, which is similar. However, bead formation at the 5.5% PCL concentration (and 6% PCL concentration) led me to choose the 8% PCL concentration. Although this was still useful in conducting these proof-of-concept studies, a closer approximation to native Bruch’s membrane would be ideal. Thus, using a surfactant or increasing the applied voltage after the apparatus refinement that removes bead formation is an approach that could be employed in the future.

Beyond electrospinning, there are other approaches that might be employed to better mimic the fiber morphology of the native Bruch’s membrane. One such approach is the use of molecular self-assembling systems as a reliable method to produce nanofibers. In one study, these nanofibers were assembled by non-covalent bindings of peptide or protein units [31]. Another study also suggested that a peptide self-assembly system can form three dimensional scaffolds with a uniform fiber diameter less than 100 nm [32]. Thus, although electrospinning has received significant attention in the literature, molecular self-assembling systems are gaining attention as a future direction. Indeed, peptide based self-assembling fibrils have already been applied in bone, nerve and cardiac tissue regeneration [33].

Another possible approach is the use of 3D printing technology, which has rapidly developed in recent years. 3D printing is basically an idea of creating a three-dimensional object under a computer control. 3D printing methods used in printing biomaterial device have recently been reviewed [34]. Methods of 3D printing include Inkjet 3D Printing, Fused Deposition Modeling (FDM), Selective Laser Sintering (SLS), Stereolithography and Bioprinting. Although 3D printing can usually offer topography or other desirable features to a biomaterial, the resolution limitation would be a drawback to mimic native biological structure. For example, the resolution of a popular Inkjet 3D Printing method is about 100 µm, which is three orders of magnitude greater than the fiber diameters for the Bruch’s membrane. A recent study did show a bone tissue application of 3D printed polymeric porous scaffolds in which pore size and porosity can be strictly controlled [35], but it remains to be seen if resolution can be achieved for ECM mimics. The pore
size and porosity of a material can play an important role in material mechanical properties, biodegradation rates, and drug delivery [36]. Thus, although 3D printing is not capable of fabricating nanometer scale fibers, the underlying keratin structure can be 3D printed. Additionally, bioprinting is a method from 3D printing. A recent study [37] reviewed that extrusion-based bioprinting techniques offer an approach to fabricating controlled vascular networks with sophisticated hierarchical structure.

III.2.3 Surface Modification for Improving Cellular Response to Scaffolds

In native tissues, cells attach to extracellular matrix (ECM) through integrin binding. Absence of binding domains on synthetic polymer scaffolds affect cell attachment in an unfavorable fashion. To improve cell attachment on hydrophobic PCL fibers on the top of fibrous-aerogel scaffold, I used a Matrigel adsorption to modify the PCL fibers to improve cell attachment. Matrigel is a protein mixture secreted by mouse sarcoma cells which includes proteins from the mouse ECM [38]. Although Matrigel has commonly been used to support stem cells [39], the undefined nature of Matrigel can create experimental variations and may lead to challenges in the use of Matrigel in FDA-approved applications. Another issue with Matrigel is that it is not known which protein(s) in the mixture actually plays an active role in promoting cell adhesion or which protein may lead to toxicity after interactions with the scaffolds material. Therefore, using defined proteins to replace Matrigel as the adsorption material is a way to make the design more repeatable. Moreover, many ECM composites can be good candidates and studies have shown the feasibility in the polymeric surface adsorption process. For example, laminin [40], fibronectin [41-42] and vitronectin [43] are all known to play important roles in the ECM. And in the study by Priglinger, et al., TGF-β2–induced RPE cells showed adhesion and migration on a matrix of pure fibronectin [42]. Based on these findings, it is clear that the fibrous-aerogel scaffold system could be used to investigate the roles of individual proteins or combinations of proteins and their effects on cell behavior.

In addition to protein adsorption treatments, there are many other surface modification methods that can be adopted to realize the same goal. As I discussed above, the use of PCL and gelatin blends as electrospinning polymeric solution is one promising possibility. Similar combinations like PCL-chitosan [44-45] or PCL-collagen [46] that blend PCL with natural
polymers to do electrospinning have been also been reported in the literature in the past. Further, plasma treatment using oxygen or other organic compounds to offer oxygen-bearing groups or amino groups may be beneficial [47-48]. A study by Prabhakaran., et al. showed that a higher cell proliferation was achieved on plasma-treated PCL electrospun fibers compared to PCL-collagen meshes [48]. Chemical reagent treatment, which has a similar effect as plasma treatment, is achieved by immersing PCL fibers into a solution to introduce hydrophilic chemical groups like carboxylate (-COOH) groups or hydroxyl functional groups (-OH). One commonly used solution in chemical reagent treatment is sodium hydroxide (NaOH). A study [49] by Serrano, et al. found that NaOH treated PCL films can retain endothelial cell growth and functionality.

**III.2.4 Drug Delivery from Scaffolds for Retinal Regeneration**

In this fibrous-aerogel scaffold design, apart from acting as the mechanical support, both keratin and PCL have been used for controlled release of various small molecule drugs and growth factors [50-51]. In terms of a tissue engineered retina, molecules of interest from a drug delivery standpoint may be exogenous agents (i.e., drugs or small molecules inhibitors) to promote cell proliferation and differentiation for therapeutic approaches, or they may be endogenous molecules that are used directly from extracts or prepared in recombinant form (e.g., recombinant growth factors). Both small molecule drugs and growth factors would be of interest in a scaffold-based in vitro model system in order to understand the roles of various molecules in disease or regeneration states and the effects of their timed delivery. The current study in seeding an RPE cell line (ARPE-19 cells) is only to investigate the basic biological utility of the scaffold system such as cell viability and cell attachment in an *in vitro* setting. However, a study of pluripotent stem cells such as human embryonic stem cells (hESc), would be a possible future step to further develop these scaffolds towards a tissue engineered retinal transplantation. In this case, growth factor delivery [52] or therapeutic gene delivery [53] in the scaffold system might be used to induce stem cell proliferation and/or differentiation with the goal of restoring native tissue function. Since the time and role of growth factor or inhibitor delivery in the cell differentiation process is complex and not fully understood, particularly for neuroretina formation, this scaffolding system allows for the application of timed drug delivery to investigate the roles of individual or combinatorial growth factor and inhibitor strategies. Following another approach, it has been suggested [54] that rapid vascularization is the minimum requirement for cells to survive after transplantation. Therefore,
vascular endothelial growth factor (VEGF) or other molecules that promote rapid and stable local vascularization is an important aspect of tissue engineering designs for retinal and other tissues [55-56]. Such strategies for vascularization could be employed upon implantation of a scaffold-cell system for therapeutic approaches or could be used to investigate dose-related effects of, for example, VEGF on vascular cells in the keratin region, as discussed above.

Although most biodegradable polymeric systems are capable of drug delivery, a review study by Nicolas et al. stated that uncontrolled drug release from drug carriers can lead to poor clinical outcomes [57]. Another study showed that a tunable drug release profiles can protect therapeutic drugs from rapid degradation or distribution to off-target sites both in vitro or in vivo [58]. Furthermore, in native retinal tissue, vascularization is accomplished by participation of angiogenic growth factor like VEGF. As I discussed in Chapter I, over-secretion of VEGF can lead to wet AMD symptoms associate with abnormal angiogenesis. As noted above, however, vascularization is a necessity for healthy retina, whether native or a tissue engineered construct. As such, a tunable VEGF release profile is needed so as to develop healthy vasculature without promoting an abnormal vasculature that can lead to AMD. From a previous study with keratin as the drug reservoir [52], it has been shown that erosion rate of the keratin hydrogel and the growth factor release from the keratin hydrogel can be controlled, suggesting the possibility of such an approach with the fibrous-keratin aerogels in the future. Since the structure of the fibrous-keratin aerogel is different than keratin hydrogel, a separate drug release study would be to determine its tunability for drug delivery.

III.2.5 Alternative Materials in Fibrous-Keratin Aerogel Scaffold Fabrication

The fibrous-aerogel system was designed to provide a combination of a nanofiber surface to promote cell attachment, growth, and proliferation with a porous scaffold to provide mechanical support and, in the future, to achieve controlled release of therapeutic molecules. Theoretically, many materials can be used to fabricate the fibrous and porous components of this scaffold system. In previous chapters, I have discussed potential materials in fabricating overlying nanofibers and found that compared to natural polymers, synthetic polymers like PLGA, PLA, PCL [59-60] have slower degradation rates that can potentially allow a longer time frame for retinal tissue regeneration to occur on the surface of the scaffold. Additionally, blends of PCL with natural
polymers were also discussed earlier as an approach to improve cell attachment. For the initial proof-of-concept studies of this thesis, PCL was a rational material selection for fabrication of the nanofibers, and these PCL fibers worked well for the fibrous-aerogel system. However, it is not known how long the fiber system should remain intact in order to best promote retinal regeneration. For example, shorter times may be needed if all cellular components can be incorporated into/onto the scaffolds during the fabrication process. However, according to a study [61] by Osakada, et al., it generally takes 90 to 150 days for human embryonic cells to differentiate to RPE or photoreceptors. Thus, materials that persist for at least three months may be more favorable for these longer-term studies.

The materials for the underlying porous structure in fibrous-aerogel system can also have a large variety of options. According to the experimental results, keratin has shown many benefits as the material in fibrous-aerogel system including tunable mechanical properties that are in the same compressive modulus range as the combination of native choroid and Bruch’s membrane. The keratin aerogel was also not observed to be toxic to ARPE-19 cells, which is clearly a key requirement of the design. However, from the compression test results, the standard deviation of the compressive modulus for each concentration were fairly large, indicating some variability in scaffold fabrication by the current preparation technique (lyophilization following the gelation of the keratin in plastic tubes). Therefore, it may be beneficial to refine the fabrication process or find alternative materials to replace keratin with materials that can be prepared in a more reproducible manner.

Although I referred to this scaffold system as a fibrous-aerogel, in the in vitro and in vivo environments, the aerogel reabsorbs water to become a hydrogel. The reason for using an aerogel instead of a hydrogel was to prevent the vertical movement of keratin in hydrogel form, which is likely to occur due to the shear thinning nature of keratin hydrogels in a horizontal electrospinning set-up. However, if a vertical system were used for electrospinning, a keratin hydrogel could be used, offering some potential advantages as a mechanical support and drug carrier. As noted previously, this approach would allow a chance to culture vascular cells in the hydrogel during electrospinning, although approaches to enhance the sterility of the system (e.g., placing the unit in a laminar flow hood) may be required.

As is mentioned in chapter III.1.1, studies have shown that mechanical properties of a substrate material have a significant influence on cell cytoskeletal organization [6-9], which can
impact cell growth and migration. It has been suggested that hydrogels have made great progress in drug delivery field over the past decade, but their poor mechanical performance has created limitations [62]. This is particularly challenging in orthopaedic applications because hydrogel scaffolds like gelatin, agarose, and alginate [62-63] cannot achieve the elastic modulus of most native musculoskeletal tissues such as cartilage [64], muscle [65], or bone [66]. However, I have not found compressive modulus data for hydrated Bruch’s membrane, so hydrogels with relatively weaker mechanical properties may be suitable for retinal tissue regeneration.
References


