Biofabrication of choroid-retina tissue construct for modelling of age-related macular degeneration disease

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**NANYANG
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**Biofabrication Of Choroid-Retina Tissue Construct for
Modelling of Age-Related Macular Degeneration
Disease**

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SCHOOL OF MECHANICAL AND AEROSPACE ENGINEERING

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Biofabrication Of Choroid-Retina Tissue Construct for Modelling of Age-Related Macular Degeneration Disease

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ABSTRACT

Age-Related Macular Degeneration (AMD) is a major cause of blindness affecting everyone above the age of fifty. The main pathological effect is the uncontrolled growth of blood vessel below the retina layer which results in neovascularisation and leaked fluid within the Bruch's Membrane. Till date, there is no cure and limited treatment options to treat AMD. There is a need for a realistic and biomimetic choroid retina tissue model to enable in-depth investigation of the diseases and to enable speedy development of new treatment options. In this project, a choroid-retina tissue model was fabricated. The Choroid-Retina tissue is made of two distinct tissues layers consist of different types of cells with different requirements, properties and functions. The layer of retina cells should present hexagonal morphology on an ultrathin substrate; while the choroid layer must be in 3D, at high resolution and interconnected. In this project, 2 different biofabrication processes were used to develop the biomimetic Choroid-Retina Tissue Construct for Modelling of Age-Related Macular Degeneration Disease. The first step involves the development of an ultrathin and porous membrane to mimic the native Bruch's Membrane for supporting a layer of functional retinal pigmented epithelium (RPE) cells. The membrane was produced through solution drop casting of a polycaprolactone/polyethylene glycol (PCL/PEG) solution. The RPE cells were cultured over a period of 15 days. The cells were able to form an epithelial barrier function on the 3rd day of culture similar to those grown on a PET (Polyethylene Terephthalate) membrane. The process is simple and can yield an

ultra-thin, porous and free-standing membrane. This membrane was shown to be able to elicit RPE cell's response and filtration properties that mimics a Bruch's membrane. The second step involves the development of a 3D choroidal capillary vasculature via bioprinting. Bioprintable hydrogel materials were formulated and evaluated for optimal biocompatibility with endothelial (HUVECs) cells. An innovative printing strategy was optimised further so that the natural occurring hydrogel with extremely low viscosity such as collagen could be printed into a free-standing 3D construct. As a result, vasculature design feature with resolution of 100 μm was achieved for soft hydrogels such as Gelatin Methylacrylate (GelMA) and Collagen Type I. The novel printing approach relies on the use of surface interaction between the primary hydrogel (collagen or GelMA) and an bioinert secondary hydrogel, Pluronic, to create self-limiting swelling in order to achieve high resolution printing. A bioprinted 3D collagen construct of 1.5mm thickness, with pre-determined pore size of 400 μm and strut size of 100 μm , was seeded with HUVECs and cultured for 12 days. The experimental results indicated that the endothelial cells (HUVECs) was able to vascularise and was functional as demonstrated by the production of platelet endothelial cell adhesion molecule-1 biomarkers (CD31) and vWF (von Willebrand Factor). The 3D structure was produced to serve as a matrix for the formation of capillary structures.

Lastly, the feasibility of creating a sandwich retinal-photoreceptor construct by combining the ultrathin membrane and bioprinted hydrogel was demonstrated. A hybrid 3D construct was created consisting of RPE cell-seeded ultrathin membrane and the photoceptor-like cells Y79. In summary, this work presents

a novel hybrid approach with different biofabrication strategies. The resultant tissue construct could be used a possible tissue model to be employed in pharmaceutical companies for drug testing of AMD patients.

List of Publications

Journals

1. Y.S.E. Tan, P.J. Shi, C-J Choo, A. Laude, W.Y. Yeong Tissue engineering of retina and Bruch's membrane: a review of cells, materials and processes, British Journal of Ophthalmology, 2018
2. Y.S.E. Tan, S. Agarwala, Y.L. Yap, C.S.H. Tan, A. Laude, W.Y. Yeong, Novel Method for The Fabrication of Ultrathin, Free-Standing and Porous Polymer Membranes for Retinal Tissue Engineering, Journal of Material Chemistry B, 2017
3. Y. S. E. Tan, W.Y. Yeong, Concentric Bioprinting of Alginate-Based Tubular Constructs Using Multi-Nozzle Extrusion-Based Technique, International Journal of Bioprinting, 2015
4. R. Suntornnond, Y. S. E. Tan, J. An, C. K. Chua, A Mathematical Model on the Resolution of Extrusion Bioprinting for the Development of New Bioinks, Materials, 2016
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7. P.J. Shi, Y.S.E. Tan, W. Y. Yeong, A. Laude, Hybrid three-dimensional (3D) bioprinting of retina equivalent for ocular research, International Journal of Bioprinting, 2017

8. P.J. Shi, Y.S.E. Tan, W. Y. Yeong, H.Y. Li, A. Laude, A bilayer photoreceptor - retinal tissue model with gradient cell density design: A study of microvalve - based bioprinting, Journal of Tissue Engineering and Regenerative Medicine, 2018
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2. J. M. Lee, W.L. Ng, Y.S.E. Tan, W. Y. Yeong, Bioprinting for Tissue Engineering, International Bone-Tissue Engineering Congress, Bone-Tec 2013, Singapore, 16-19 December 2013 (Poster)
3. Y. S. E. Tan, W.Y. Yeong, Direct, Bioprinting of Alginate-Based Tubular Constructs Using Multi-Nozzle Extrusion-Based Technique, 1st International Conference on Progress in Additive Manufacturing, ICPRO-AM 2014, Singapore, 26-28 May 2014 (Oral)
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Book Chapter

1. J.M. Lee, Y.S.E. Tan, Z.C. Zhu, W.Y. Yeong (2015) Biomaterials for Bioprinting. In L.J.G Zhang, J. Fisher, K. Leong, 3D Bioprinting and Nanotechnology in Tissue Engineering (1st Edition)
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Patent

1. R. Suntornnond, J. An, C.K. Chua., Y.S. E. Tan, W.Y. Yeong and J.K. Er, "Free Standing Hollow 3D Bioprinted Structure from Highly Printable Bio-Ink", Singapore Patent, Provisional Application No. 10201701620X

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

14/10/2019

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Date

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Tan Yong Sheng Edgar

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

14/10/2019

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Date

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Assoc. Prof. Yeong Wai Yee

Authorship Attribution Statement

This thesis contains material from 4 paper(s) published in the following peer-reviewed journal(s) / from papers accepted at conferences in which I am listed as an author.

Chapter 2 is published as Y.S.E. Tan, P.J. Shi, C-J Choo, A. Laude, W.Y. Yeong Tissue engineering of retina and Bruch's membrane: a review of cells, materials and processes, British Journal of Ophthalmology, 2018

The contributions of the co-authors are as follows:

Assoc. Prof. Yeong Wai Yee, Dr C-J Choo and Dr Augustinus Laude edited the manuscript drafts.

I prepared the manuscript, performed all the laboratory work at the School of Mechanical and Aerospace Engineering and analysed the data.

Dr Shi Pu Jiang assisted in the collection of data and provided guidance in the interpretation of the cell viability measurements.

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I prepared the manuscript, performed all the laboratory work at the School of Mechanical and Aerospace Engineering and analysed the data.

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Assoc. Prof. Yeong Wai Yee, Dr H.Y. Li, Dr Augustinus Laude edited the manuscript drafts.

Dr Shi Pu Jiang and me prepared the manuscript, performed all the laboratory work at the School of Mechanical and Aerospace Engineering and analysed the data.

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List of Abbreviations

AMD	Age Related Macular Disease
apoB	Apolipoprotein B
ATR-FTIR	Attenuated total reflectance Fourier transform infrared spectroscopy
BM	Bruch's Membrane
CAD	Computer aided design
CNV	choroidal neovascularization
Col-I	Collagen type I
DMEM	Dulbecco's Modified Eagle's Medium
DPBS	Dulbecco's Phosphate Buffered Saline
ECM	Extracellular matrix
EDC	N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
EL	elastin layer
EthD-1	Ethidium Homodimer
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FTIR	Fourier transform infrared spectroscopy
GelMA	Gelatin methacrylate
H&E	Haematoxylin and eosin
hESCs	human Embryonic Stem Cells
HSCs	Hematopoietic stem cells

HUVECs	Human umbilical vein endothelial cells
ICL	Inner Collagenous Layer
iPSCs	induced Pluripotent Stem Cells
MMP	matrix metalloproteinase
MSCs	Mesenchymal Stem Cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWCO	Molecular weight cutoff
OCL	Outer Collagenous Layer
PBS	Phosphate buffer saline
PCL	Polycaprolactone
PEG	Polyethylene glycol
PET	Polyethylene Terephthalate
PLGA	Poly(lactide- co-glycolide)
PLLA	Poly(L-lactic) acid
PNIPAAm	Poly (N-isopropylacrylamide)
PVA	Polyvinyl alcohol
RPE	Retinal Pigmented Epithelium
SDS	Sodium Dodecyl Sulfate
SEM	Scanning electron microscope
TE	Tissue engineering
T _g	Glass Transition temperature
UCST	Upper critical solution temperature
UV	Ultra violet

VWF von Willebrand Factor

XG Xanthan Gum

Chapter 1 Introduction

1.1 Background

The Age Related Macular Disease (AMD) is the leading cause of blindness in the elderly worldwide¹. AMD is caused by degeneration of the central region of the retina known as the macula due to age related changes in Bruch's Membrane (BM) and the Retinal Pigmented Epithelium (RPE)². Although comprising only four percent of the retinal area, the macula is the only region of the retina capable of 20/20 vision, and lesions here can severely affect central vision as well as quality of life. AMD can be classified into two forms, each with a different underlying pathological cause: 1) presence of drusen and cumulative damage to the RPE and Bruch's membrane leading to the degeneration of overlying photoreceptor cells ("dry" AMD), and it is typically a slowly progressing disease. However geographic atrophy represents the late, severe form of dry AMD³; 2) A more rapidly progressing form of AMD due to choroidal neovascularization (CNV) leads to haemorrhage and fluid leakage, and eventually photoreceptor damage ("wet" AMD), and it can further progress to form a disciform scar, fibrotic scarring that causes a catastrophic loss of central vision⁴.

There is currently no cure for AMD. Several options are available to slow the progression of the disease, including injection of anti-angiogenic (anti-VEGF) medications or the use of argon laser. Current research focuses on the transplantation of RPE-choroidal tissue and RPE cell. However, transplantation of these cells is relatively new and is currently still under clinical trial.

As growing of native retinal structures are relatively challenging, new material fabrication and tissue engineering approaches are utilized to create an artificial BM scaffold for sub-retinal replacement with full functions. These approaches include cell-sheet engineering⁵, decellularized membrane⁶, tissue-engineered membranes^{7 8} and nanofiber membranes .

Despite the importance of the retina and the effects of AMD on the retina tissue, the interaction between RPE cells and the underlying retina capillaries tissues in healthy and diseased retina has not been fully understood⁹. The fundamental pathology of this complex degenerative disease is incompletely studied, including its genetic as well as epigenetic risk factors. This is partially due to the limitation of animal models¹⁰⁻¹² and *in vivo* models¹³. The *in vivo* models involve the complexity of the environments, differences between human and animal retinal tissues and the challenges in studying the embryonic retina cells. These drawbacks highlight the research need create a realistic and biomimetic *in vitro* model.

In vitro retina tissue models have unique advantages for studying cellular interaction by simplifying the complexity of an *in vivo* environment, improving accuracy of model by utilising human cells and cost-effective when compared to animal and human studies. Current reported retinal tissue models consist mainly a 2D layer of retinal pigmented epithelial cells on substrate. There is limited or no prior report of a 3D retinal model co-cultured with photoreceptors and blood vessels to resemble a complete 3D structure of the retina.

1.2 Objectives

This research intends to develop a tissue model to mimic the retinal tissue configuration and design features. The objectives of this thesis are:

- Development of an ultra-thin biocompatible polymer membrane to simulate Bruch's membrane for adhesion of RPE cells.
- Development of novel process for indirect bioprinting of supported hydrogel bioprinting for vascularization.
- Prove-of-concept using hybrid bioprinting and membrane fabrication to produce retinal tissue complex.

1.3 Scope

The scope of the project is as follows:

- Developing a novel fabrication process for biomimetic ultra-thin membrane with comparable properties as natural bruch membrane.
- Developing a functional layer of retinal cells on the ultrathin membrane.
- Studying the surface interaction of hydrogel for bioprinting.
- Establishing support hydrogel's bioprinting process.
- Determining feasibility of using surface interaction to improve the strength of bioprinted hydrogel.
- Characterizing of supported hydrogel properties for in vitro cell culture study.
- Proof-of-concept for fabrication of 3D retinal tissue complex.

1.4 Organization of Thesis

This thesis begins with an introduction (Chapter 1) which includes background, objectives and scope of this project.

Chapter 2 covers the literature review of hydrogel's behaviour and current trends of tissue engineering of retinal tissue.

Chapter 3 presents the fabrication of a synthetic ultra-thin membrane.

Chapter 4 presents a feasibility study on using surface interaction to strengthen bioprinted hydrogel structure.

Chapter 5 describes a developed version of a supported hydrogel printing system.

Chapter 6 presents the proof-of-concept for fabrication of 3D pigmented human retina complex using phase separation and bioprinting technique.

Chapter 7 concludes the current research and proposed the future research plans.

Chapter 2 Literature Review

2.1 Retina Anatomy and Physiology

2.1.1 Bruch's Membrane

Bruch's Membrane (BM) is a unique five-layered extracellular matrix that sits at a critical juncture between the RPE and the choriocapillaris¹⁴, and it is composed mainly of elastin and collagen fibres and acts as a semi-permeable filter, facilitating the metabolic exchange of biological molecules between the retina and the choroid¹⁵. Nutrient material passes from the choriocapillaris to the RPE, while cellular waste from the RPE travels in opposite directions¹⁶. BM has major clinical interests because it involves in development of AMD and other chorioretinal diseases.

BM ultrastructure

Hogan identified five unique ultrastructural layers in BM in the early 1960s¹⁷. These are the basement membrane of the choriocapillaris, the outer collagenous layer (OCL), the elastin layer (EL), the inner collagenous layer (ICL), and the basement membrane of the RPE, which represents the innermost layer (**Figure 2-1**). The basement membrane of the choriocapillaris, at approximately 0.14 μm in the young, is made up of collagens type I, IV, V, and VI, laminin and heparan sulphate. Type IV collagen, which is predominantly found as the structural component of the choroidal basement membrane, may play an active role in securing BM to the capillary endothelial cells of the choroid¹⁸. The EL is composed of elastin fibres that together form a perforated sheet with interfibrillary spaces of about 1 μm . Additionally, the EL also contains collagen

type VI and fibronectin, and other proteins¹⁹. Chong et al. found that the EL is up to six times thinner and exhibited a higher degree of porosity (up to five times) in the macula region as compared to the periphery of the retina, a possible explanation for the increased incidence of choroidal neovascularization (CNV) at that region²⁰. The ICL is approximately 1.4 μm thick, while the OCL is generally thinner at 0.7 μm in the young. The collagen in these two layers are 65 nm striated collagen fibrils I, III, and V. The type I collagen is thought to confer tensile strength to tissue, and the type III confers elastic properties, and type V facilitates the adhesion of the basement membranes of the RPE and choroid to the collagenous layers²¹. Both are otherwise largely identical, and their collagen fibers are arranged in a grid-like fashion. This grid is saturated with other matrix components such as glycosaminoglycans (chondroitin sulphate, dermatan sulphate and hyaluronic acid), and components of the coagulation and complement system²². The basement membrane of the RPE is approximately 0.15 μm thick in the young eye, and is said to resemble in many ways the basement membranes found in other parts of the body.^{23 24}. For example, both the basement membranes of the RPE and the kidney glomerulus contain collagen IV α 3–5, possibly important in their specialized filtration and transport functions²⁵. On the other hand, while the basement membrane of the RPE is largely similar to that of the choriocapillaris, which does not contain type VI collagen. Aisenbrey et al. found that the RPE synthesizes specific integrins that bind to laminins and type IV collagen in the RPE basement membrane, attaching the RPE to BM^{19 26}.

Figure 2-1: Detailed Bruch membrane and RPE structure

Molecular structure of BM

BM is an acellular matrix that relies on adjacent structures (RPE and choroid) to produce most of its constituent components. Of the proteins identified in BM, the majority is produced by the choroid, supporting the suggestion²⁷ of a common developmental origin of BM and the vascular intima of the choriocapillaris.

Figure 2-2: A basic schematic of the disease pathway of AMD on a healthy retina tissue

Changes of BM, choroid and RPE with age, and the pathogenesis of AMD

Histological and chemical changes occur in aged BM (**Figure 2-2**). The average thickness of BM increases with age, nearly doubling in size with the deposition of proteins, extracellular lipids, as well as other waste products²⁸⁻³⁰. Evidence of such accumulations can be seen as early as 10 years of age³¹. By employing quick-free/deep-etch methods, Ruberti et al. identified the accumulation of esterified cholesterol and unesterified cholesterol in BM with age³⁰. These lipids are deposited in a discrete sublayer external to the RPE basement membrane in the eyes of many of those older than 60 years of age³². The origin of these lipid particles is unclear, although its molecular makeup points to a cellular origin, as opposed to being derived from blood plasma from the choriocapillaris. Indeed, the RPE is capable of synthesizing apolipoprotein B (apoB), a major component of very-low-density lipoproteins and intestinal chylomicrons, and this can be

detected in Bruch's membrane, suggesting that the RPE is the likely source of these lipid accumulations³³. Starita et al. demonstrated that the hydraulic conductivity of Bruch's membrane decreases with age³⁴. Undeniably, extrapolated data by Fisher showed that flow of water through BM is expected to be reduced to nil by age 130³⁵. This is consistent with the formation of a lipid wall, rendering BM increasingly hydrophobic and impermeable to hydrophilic substances. Retinoid derivatives from the choroidal circulation cannot reach photoreceptors, and perhaps contribute to photoreceptor dysfunction and the pathogenesis of AMD³⁰.

Collagen cross linkages are also thought to occur in BM as it ages, and this too has a direct effect on the elasticity and permeability the membrane. The dense collection of collagen fibres also proves a difficult barrier for the passage of RPE collagenases, resulting in an impaired turnover of BM components³⁶. Important glycoproteins found in BM, such as heparan sulphate, lose the ability to perform key regulatory functions such as processing its core protein. Heparan sulphate is thought to regulate the complement cascade, and its malfunction may be one of the critical molecular switches that turn normal BM ageing into AMD pathology^{22 37}. Further calcification also occurs due to aging of BM, rendering the membrane brittle and predisposed to breaking and forming neovascularization, and was demonstrated by Spraul et al. as having a positive correlation with AMD³⁸. Iron also deposits, leading to damage from oxidative stress, and the accumulation of advanced glycation end products (AGEs) causes age-related damage in BM and choroid ³⁹⁻⁴².

The appearance of drusen and pigmentary changes in the retina marks some of the earliest signs of AMD in the eye. Large druse is the hallmark of AMD and appears as focal deposits of polymorphous, extracellular material between the basement membrane of the RPE and ICL of BM. Not all drusen are associated with AMD. Cuticular or basal laminar drusen, or drusen smaller than 63 μm are not considered indicative of AMD. Focal hyperpigmentary changes in the RPE also commonly appear in AMD, and these are seen as focal areas of grey or black pigment⁴³. Drusen typically occurs in the region of the macular, likely due to the high density of photoreceptors here which leads to a localized region of oxidative stress⁴⁴. Waste products from the macular RPE traversing BM to be removed by the choroidal circulation may undergo oxidative modulation and deposit in BM. The same is true for nutrients brought in by the choriocapillaris heading to the RPE. This model of oxidative stress leading to AMD pathology is supported by findings that transgenic mice susceptible to oxidative stress showed signs of retinal degeneration that closely resembled the changes seen in AMD^{45 46}.

2.1.3 The Retinal Pigment Epithelium

The RPE is a highly metabolically active structure that lies between photoreceptor cells and BM. The RPE is vital to the survival and function of overlying photoreceptor cells, serving a variety of metabolic and supportive functions, such as the transport of nutrients and solutes from the choroidal circulation towards the photoreceptor layer. The cells of the RPE are tightly connected to each other by adhesions and junctions, forming a monolayer of cells that act as the blood-retina barrier ⁴⁷. It also plays a role in absorbing stray

light, phagocytosing the photoreceptor outer segment (POS) debris that is shed by cone and rod cells, and the recycling of retinol in the visual cycle^{48 49}.

The adhesion of the RPE to BM is controlled by integrin cell surface receptors, found on the surface membrane of the RPE directly opposing BM. These integrin molecules bind to laminin and type IV collagen found in the matrix of BM, forming “anchoring plaques”⁵⁰. Fang et al. (2009) found that RPE cells that were cultured to over-express integrins displayed increased adhesion to BM. *In vitro* wound healing is disturbed if integrins are inhibited⁵¹. RPE wound healing appears to be more effective in the presence of the RPE basement membrane found on BM. In a study by Wang et al. (2003), debridements were created in RPE-choroid-sclera explants, exposing either the RPE basement membrane, or the ICL immediately beneath. Eleven pairs of eyes were observed for a period of 10 days to visualize RPE regrowth. The results found that while wound resurfacing occurred to some degree in all eleven pairs of eyes, eyes with the deeper ICL wounds showed less healing potential, suggesting the importance of the RPE basement membrane in RPE cellular regeneration⁵².

Pigmentary changes are seen in the RPE as it ages. Lipofuscin, a brown-yellow, electron-dense autofluorescent material accumulates as granules, and are found to be present in the basilar portions of posterior pole RPE by age 16. Although the exact origins of this autofluorescent material is unknown, it has been shown that they are mainly derived from vitamin A, and have been theorized to originate from the degradation products of photoreceptor outer segment membrane⁵³. Lipofuscin is known to generate reactive-oxygen species

(ROS) that disturbs lysosomal integrity, induces lipid peroxidation, reduces phagocytic capacity and causes RPE cell death⁵⁴. Rozanowska et al. (1995) postulated that lipofuscin acts as a photosensitizer that mediates light induced apoptosis, contributing to the development of AMD⁵⁵. Wihlmark (1997) tested this hypothesis by culturing RPE cells heavily loaded with lipofuscin and irradiating them with blue light, finding that these cells exhibited a considerably enhanced loss of viability. As mentioned earlier, lipofuscin has also been discovered to induce the complement system, which has been found to play an important role in the development of AMD⁵⁶.

The age related pathological changes of BM and surround tissue play substantial roles to drive the development of AMD. In order to restore the full function of BM, research and development of tissue-engineered retinal replacement is considered as effective manners. Selections of cells, materials and fabrication techniques are vital factors for successful tissue engineered retina.

2.2 Retina Tissue Engineering

2.2.1 Retinal Cell source

For a successful retinal epithelium repair, the cells used to replace the retina must be delivered into the subretinal space⁵⁷. These cells have to be connected to the host's remaining visual synaptic pathway to repair and restore vision⁵⁸. To aid this step, researchers have to accurately identify the correct components that will elucidate the specific signals for such a repair to take place⁵⁹. Although research has shown that the delivery of a healthy cell population into the subretinal space does indeed alleviate the early stages of the disease⁶⁰, these

cells have to be functional for the cell layers to remain active. Transplanted cells must be capable of repopulating the damaged sites. Furthermore, cells must integrate into the host's retinal neuron layer and re-establish correct cellular organization and synaptic connections⁶¹. If the photoreceptor layers are damaged, these cells should also be able to differentiate towards a photoreceptor lineage with functional outer segments sensitive to light stimulation. Multiple cell types have been studied in an effort to investigate the repair mechanism of the retinal tissue⁶².

Mature retinal cells

The transplantation of RPE cells has been tested in clinical trials⁶³. Autologous cells were harvested from the patient's ipsilateral eye and implanted onto the damaged sites. These transplanted RPE cells have led to an improvement in visual acuity as compared to the control. The results provided evidence the transplantation of mature RPE cells themselves are reasonable treatment methods for AMD. Several studies have also been done on reprogramming RPE cells to differentiate into retinal neurons by upregulating the transcription factors such as Sox2 and neurogenin-1⁶⁴. Such reprogramming of RPE cells may offer an approach in repopulating the neural retina in the future. However, the surgical procedures required to harvest the cells and manipulate within the neural retinal hole is challenging, especially to separate the RPE cells without disturbing the choroid region⁶⁵. Furthermore, the applications of non-optimal aged RPE cells may incur a certain level of scepticism over the competency of the cells. Besides, autologous cells tend to contain genetic similarities that might lead to reoccurrence of the disease.

Unipotent stem cells

A subpopulation of RPE cells has been reported to be able to dedifferentiate into RPE stem cells, losing its RPE cell markers, the cells are able to proliferate better than its mature cells and can later be re-differentiated into its stable RPE monolayer⁶⁶. The use of such methods could make available a much more potent source of retinal replacement cells.

Multipotent stem cells

Multipotent stem cells such as HSC (Hematopoietic stem cells) and MSC (Mesenchymal Stem Cells) have been used in treatments to improve the retinal circulation and to enhance the survival of the outer retina⁶⁷. The cells are injected into the patient's retina as a form of regenerative therapy⁶⁸. However, due to high costs and the requirement of a multidisciplinary infrastructure to obtain the cells and the related high mortality and morbidity rate, such transplantation may not be viable.

Pluripotent stem cells

Two heavily researched cell lines that could have a huge impact on tissue engineering are human embryonic stem cells (hESCs)⁶⁹ and induced pluripotent stem cells (iPSCs)⁷⁰. hESCs are derived from the inner cell mass of blastocyst, and have the potential to differentiate into any lineage of mature cells. Clinical studies have shown that the use of hESCs-derived RPE cells for patients with dry AMD and Stargardt's AMD resulted in no signs of hyper-proliferation, abnormal growth or immune rejection in the patients⁷¹. Vision improvement was observed for those with dry AMD. Thus, these embryonic stem cells (ESCs) can

be considered for regenerating damaged RPE cells that extend to the neurons. However, the ethical issues regarding ESCs and the risk of formation of teratomas within the retina must be overcome and safety procedures have to be established before such cells can move forward and be used as part of a routine clinical procedure⁷².

iPSCs on the other hand are derived from adult cells that have been transfected with four specific transcription factors - OCT4, Sox2, cMyc and Klf4⁷³. After transfection, the cells were reported to have obtained pluripotency. Specifically, they developed the ability to differentiate into cell types not limited to the cell's previous lineages. For example, these cells have been used to generate ganglion-like cells and photoreceptor cells. These cells are good candidates for cell replacement therapy in cases where AMD has damaged the neural cell layers.

2.2.2 Tissue Engineering Approaches

Cell Sheet

Cell sheet engineering has been popular in tissue engineering research recently. The principle is based on the cells secreting its own ECM upon reaching confluency and it can be harvested without the use of any enzymatic methods⁷⁴. The ability to harvest these cells is based on the thermoresponsive coating on the culture dish. The dish switches its hydrophobicity of the surface. Such an approach allows the formation of an undisturbed monolayer of cells. The same process can be modified to improve and produce patterned substrates of thicker layers⁷⁵. However, limitation in this technology appears when the

construction of hypocellular tissue such as the Bruch's membrane is unlikely as the amount of ECM secreted by the cells is limited. Thus the formation of a mechanically strong tissue is still unlikely to be fabricated by the cell sheet technology.

Scaffolds

Like all tissue engineered scaffolds, the retinal scaffolds should be made of biocompatible materials that do not induce autoimmune response⁷⁶. It must also be mechanically stable to withstand surgical manipulations and flexible to avoid damaging the tissues around the sub-retinal region⁷⁷. The scaffold must be also extremely thin to accommodate the sub-retinal space (5-90um). A wide variety of scaffolds that has been examined by researchers are listed in **Table 2-1**. The scaffolds were categorized based on materials and fabrication techniques. The advantages and disadvantages of each approach are presented.

Figure 2-3: Retina Membrane-based TE approaches

Synthetic or naturally derived biomaterials based scaffolds (**Figure 2-3**) support cell attachment and tissue development^{78,79}. These scaffolds can also aid in directing cellular behaviors, and in the delivery of drugs or cells into the body. There are multiple approaches to constructing these scaffolds. One of the most direct methods is using a decellularised membrane⁸⁰. These membranes are extracellular matrix made by elimination of cellular components from autologous tissues. Some examples of naturally occurring membranes that have been considered for implantation into the sub-retinal space are the human amniotic membrane⁶, lens capsule⁸¹ and BM⁸². These scaffolds are especially biocompatible and mimic the natural mechanical properties of the damaged sites. Nevertheless, there are still problems with availability and donor

limitations, and also concerns over the transmission of diseases from the donor to patient.

Natural materials scaffolds

Fabricated scaffolds from synthetic or natural sources have also been considered for a range of scaffold applications including for use in the retinal region^{83 84}. Naturally occurring polymers such as collagen, alginate and fibrin have been used as hydrogels and films in almost all tissue engineering fields. Collagen, a naturally derived primary component of extracellular matrix (ECM) that makes up 25-35% of the body's protein content, has type I, III, IV and V representing the major components of the Bruch's membrane⁸⁵. It is widely investigated as their mechanical properties⁸⁶ and degradation⁸⁷ can be tuned via various methods. However, as with most natural occurring gels, it is difficult to control the consistency of these products and the mechanical properties of these scaffolds. There are also concerns over the purity of these naturally derived polymers providing animal to human disease transmission and allergy reaction. These concerns are motivating the development of synthetic materials scaffolds.

Synthetic materials scaffolds

Much better control can be exerted over the properties of scaffolds fabricated using synthetic polymers⁷⁸. Properties such as degradation, biocompatibility, melting point, transitional temperature and mechanical strength can be built directly into their chain. Polymers as such can be manipulated to degrade under very specific conditions such as pH⁸⁸ and temperature⁸⁹, depending on its

intended application. One such example of a synthetic polymer is poly(lactic-co-glycolic) acid (PLGA)⁹⁰. The polymer degrades via hydrolysis of the ester linkages into lactic and glycolic acid which can be further degraded in the body as they are common components for metabolism in cells during the Cori cycle⁹¹. By varying the ratio of the lactic and glycolic acid subunits in the PLGA polymer, the degradation of the polymer can be controlled⁹². Although these scaffolds are adaptable to different conditions, they still have to be manipulated to mimic the mechanical and biological properties of soft tissues.

Table 2-1: Membrane Scaffolds in Retinal Tissue Engineering

	Materials	Method	In-vivo /In-vitro	Cell Type	Architecture	Result	Ref.
Scaffoldless							
	N-isopropyl acrylamide	-	In -vitro	ARPE-19	Formed monolayer cell sheet	Different growth factors affect the formation and shrinkage of monolayer	93
	Magnetite	seeded RPE cells uptake with magnetic nanoparticles	In-vitro	ARPE-19	Form multilayer cell sheet	Magnetically controllable cell sheet	94
Natural	Collagen film	UV-crosslinking and drying	In -Vitro	ARPE-19	2.4 $\mu\text{m} \pm 0.2 \mu\text{m}$ thick	Sufficient permeation of nutrients of media	83
	Gelatin film	EDC/glutaraldehyde crosslinked	In -Vitro	ARPE-19	30 μm	EDC crosslinked film are more stable compared to Glutaraldehyde-crosslinked films and the ability to melt allows it to be an	95

						efficient transfer and encapsulation material	
	Lens Capsule	Dried in UV lamp	In -Vitro	ARPE-19	2-28 μm thick	Stamped with PDMS to create hexagonal structure	96
	Cicada Wings	-	In -Vitro	ARPE-19	-	Hexagonal patterned nanostructure improved cell adhesion, proliferation and gene expression	97
	Blood Plasma	Cryoprecipitation	In -Vitro	HFRPE	100 μm	Fibronectin and fibrinogen content in the film improved attachment and proliferation and helped in the anchoring of integrins	98
	Amniotic membrane	-	In -Vitro	Rabbit RPE	-	AM promotes epithelial differentiation	99
Synthetic	PMMA-co-PEGM	Electrospinning	In -Vitro	ARPE-19	50 μm	A nondegradable BM mimicking membrane modifiable with	100

						short peptides that are compatible with the RPE cells	
	PCL	Electrospinning/spin coating	In-Vivo	-	50 μm	PCL membrane is tolerable in the subretinal space	¹⁰¹
	PCL	Drop-casting of PCL solution on liquid		ARPE-19	5-6 μm	free-standing scaffold with nanometer pores ARPE-19 cell mitosis and spreading observed on membrane	¹⁰²
	Polyimide micro electrode array	-	In-Vitro and In-Vivo	Human RPE	18.5 μm	Good affinity with gold coated polyimide film	¹⁰¹
	Polyester membrane	-	In-Vitro	HESC-RPE	10 μm	Expression of integrins are limited until the addition of manganese/ overexpression of kindlin-1/alpha9 integrins	¹⁰³
	PLLA /PLGA	Solvent casting	In-Vitro	Human, Rabbit and Porcine RPE	10-30 μm	Normal morphology expressed after 14 days of culture with RPE cells developing a smaller cell size until tissue layer was formed	¹⁰⁴

	PLDLA	Solvent Casting	In-Vitro	HESC-RPE	20 µm	Relatively high membrane porosity with surface coating to mimic the collagen on the Bruch's membrane	105
	Poly(ether) Sulfone	Casting	In-vitro	Human RPE and Bovine RPE	-	PEG methyl ether Methacrylates improved RPE cell functionality without the use of biologically derived matrices	106
Hybrid	PCL/Gelatin	Electrospun	In-Vitro	Human RPE	25-34 µm	Expression of RPE65 and Cytokeratin 8/18 was present but lacks	107

2.2.3 Fabrication of standalone ultrathin BM membrane

Recent published articles have shown viable methods for direct fabrication of standalone ultrathin membranes⁷⁸. Methods such as phase separation and electrospinning can produce nanoporous surfaces during fabrication which could control cell-substrate interaction. Other structural patterning methods include creating aligned fiber substrate as cell-directive substrates¹⁰⁸, and laser-induced surface topology on biodegradable membrane¹⁰⁹. These methods of fabrication can produce controllable topology by adjusting machine parameters, allowing them to produce more precise and repeatable patterns. Further studies of the novel materials that could mimic the properties of native acellular tissue such as porosity, compressive modulus, permeability and viscoelasticity could be a solution to coax retinal cell behavior, not only in the quality of ECM produced, but also in their ability to integrate with their host.

2.3 The Choroid

The choroid is a vascular layer of the eye containing connective tissues. Its main function is to supply nutrients, such as pigment precursors, lipids, essential vitamins, and oxygen that are needed to the outer retina layer. These nutrients pass through the fenestrated endothelial cells of the choriocapillaris, diffusing passively through BM towards the RPE. Flow rate through BM depends on local concentration of salts, glucose, and pH¹¹⁰, and the maximum rate of diffusion across the membrane occurs at the isoelectric point of pH 5.0. A negative charge is found on BM at physiological pH, and this may block the passage of negatively charged molecules^{22 111}.

As the choriocapillaris ages, the normally sinusoidal capillaries slowly become tubular, and there is a concurrent decrease in its cross-sectional area. The density of the choriocapillaris falls, leading to a reduction of blood flow, and a decreased clearance of RPE waste matter via the choriocapillaris circulation. It is not clear why this happens, although there is reason to believe that it is related to the thickening of BM. The thick BM with reduced permeability affects the passage of regulatory molecules from the RPE that regulates the choriocapillaris, causing the capillaries to undergo transformation to a more common tubular arrangement^{36 112}. In general, the choriocapillaries have a general size of about 50 μm in diameter and is able to withstand pressure of up to 4.6KPa.^{113 114}

2.3.1 Current Approaches for Fabrication of Tissue Engineered Blood Vessel

Gel Casting of tubular hydrogel

One of the most widely used technique to align cells within a vascular graft was the fabrication of a collagen gel compacted around a non-adhesive mandrel(**Figure 2-4**).¹¹⁵ This technique induces significant alignment of the cells but has inherent drawbacks such a technique is the

fabrication of the hydrogel structure can only produce a single tubular structure. Fabrication of complex capillary network would not be possible as this technique could only fabricate tubular structures with a singular inlet and outlet structure. Also due to the limitation in the setup, the fabrication of smaller tubular structures (<1 mm diameter) could not be produced.

Figure 2-4: Fabrication device with resulting macroscopic tubular gel. (A) Shear chamber assembled and (B) solution loaded in shear chamber. (C) Fabrication procedure showing the inner rod's combined rotation and retraction movement allowing the curing solution to flow into the lumen of the tube (D) Macroscopic photo of the final tube in its tubular form.¹¹⁵

Photopatterning of hydrogel

One of the common methods for fabrication of a 3D patterning structure is through the use of stereolithography-based technique. This approach allows great versatility and ease of construction. By restricting or directing light at specific location on a hydrogel precursor with photo initiators, the polymerising of the structures can create specific patterns which can be

controlled spatially. These techniques range from the use of DMD (digital micromirror device) projection in DLP (digital light processing) printers to SLA (sterolithography) to achieve the completed products. This process uses a precursor solution that contains a mixture of cells and polymers that contain acrylate group, and photo initiator. However, this technique generally has limitations in terms of the type of material use and the process would expose the cells to high levels of UV radiation during printing. The duration of the exposure increases with the thickness of the structure.

2.3.2 Materials Used in the Fabrication of Tissue Engineered Blood Vessel

Chitosan and Chitin

Chitin is a co-polymer of *N*-acetyl-glucosamine and *N*-glucosamine units randomly or block distributed throughout the biopolymer chain depending on the processing method used to derive the biopolymer ¹¹⁶. The biopolymer is termed chitin if the number of *N*-acetyl-glucosamine units is higher than 50%. Otherwise, the biopolymer is termed chitosan. Chitin and chitosan are commercially obtained from shellfish sources such as shrimps and crabs

Chitosan is a variant of chitin, a partially deacetylated counterpart. Chitosan is degradable by enzymes in humans and is structurally similar to naturally occurring glycosaminoglycans. Chitosan is soluble in dilute acids and once dissolved, it can be gelled by extruding the solution into a nonsolvent or increasing the pH value. Chitosan is degraded by lysozyme and the degradation kinetics is inversely proportional to the degree of crystallinity. Its degraded product includes chitosan with lower molecular weight, *N*-acetyl-D-glucosamine residues and chitooligomers ¹¹⁷.

Poly(ethylene glycol) (PEG)

PEG is a biocompatible and hydrophilic material, with properties that limit antigenicity, immunogenicity, cell adhesion and protein binding¹¹⁸. PEG homopolymer is a polyether which can be polymerised from ethylene oxide by condensation. PEG is non-adsorptive due to the lack of protein binding sites on the polymer chain. As compared to PHEMA and PVA, PEG does not have hydrogen bond donating groups, a feature that is critical in reducing protein binding.

PEG hydrogels have been considered as one of the most successful synthetic hydrogels for tissue engineering applications. The ends of PEG polymer can be modified with either acrylates or methacrylate to form photocrosslinkable polyethylene glycol dimethacrylate, PEGDA¹¹⁹. PEGDA are extensively used to encapsulate cells into scaffolds. Furthermore, PEG can act as a mediator for immobilising the RGD sequence. As tissue engineered blood vessel, PEGDA has been used significantly to encapsulate HUVEC cells in micropatterning.¹²⁰

Collagen

Collagen, a well-known protein, has been extensively used in biomedical applications as it is the main component of natural ECM and also the most abundant protein in mammalian tissues¹²¹. Although a large number of natural and synthetic polymers are used as biomaterials, the characteristics of collagen are distinct from those of synthetic polymers largely in its mode of interaction in the body. More than 20 different types of collagen have been found but their basic structures are the same, namely, consisting of three polypeptide chains. These three chains wrap around one another, thereby creating a three-stranded rope structure. The strands are held together by covalent bonds and hydrogen. Stable collagen fibres can initiatively form as a result of strands self-aggregating. Furthermore, the mechanical properties of collagen fibres can be enhanced by introducing chemical

crosslinkers such as carbodiimide and glutaraldehyde^{122 123}. Collagen is naturally degraded by metalloproteases and thus, the degradation process can be locally controlled by cells in the engineered tissue. However, manipulating collagen is relatively difficult as collagen precursor is extremely non-viscous and takes up to 30mins to completely cure. Furthermore, collagen is extremely sensitive to the changes in the chemical properties and would change their physical properties quite easily.

2.4 Emerging Technology

2.4.1 Bioprinting techniques

Figure 2-5: Different Inkjet, microextrusion, and laser-assisted bioprinting technologies. (A) Thermal and piezoelectric inkjet printing, (B) Microextrusion printers in the form of pneumatic, piston-driven, or screw-driven systems. (C) Laser-guided direct cell printing (D) Laser-induced direct cell printing.¹²⁴

Recently, a relatively new additive fabrication technique termed “Bioprinting”(Figure 2-5) has been introduced into tissue engineering¹²⁵. Compared to other microfabrication technologies such as photolithography¹²⁶ and electrohydrodynamic fabrication¹²⁷, printing resolution of a commercial bioprinter still could not achieve nanometer resolution, however bioprinting has advantages in direct cell deposition¹²⁸ and complex 3d structures¹²⁹ can still be fabricated through this technique. In current conventional bioprinter, cells can be embedded within the

hydrogel in a single step, compared to lithography, a few steps are still required to create a pattern¹³⁰. Also, current lithography techniques however, can only fabricate 2.5D structures and are more suited towards patterning of flat structures such as fluidics chip rather than complex tissues¹³¹. Moreover, bioprinting boasts the capability to accurately deposit multiple materials onto the build plate¹³². Thus, this seems to be a possible and feasible method to create a tissue complex that is able to incorporate both the RPE cells and other cell types within a single part.

These features could play an important role in BM scaffold fabrication. Bioprinted film of natural material could potentially serves as a BM substitute for retinal cell attachment^{133 134}. For example, photoreceptors and retinal bipolar cells can be bioprinted onto the retina and aligned and orientated along the curvature of the globe. Initial indications of success has been shown by other organ tissues such as the kidney¹³⁵, which has displayed important cell-cell interactions between the different cell types and the effects of signal pathways that control cell differentiation and cell morphological changes. Other approaches in tissue repairs such as gene therapy and drug delivery and targeting have been suggested as another solution to the problem. Genes have been successfully delivered into target RPE cells to restore genetic defects such as autosomal recessive retinitis pigmentosa (RP)¹³⁶ in mice. Gene therapy has also been successfully used in dogs to restore vision¹³⁷. The combinatory use of gene therapy and tissue engineered scaffolds could hopefully provide an effective solution to AMD.

Presently, most bioprinted products are being used in many applications spanning from drug test models¹³⁸ to implantable devices¹³⁹. The high precision controls in the 3D space combined with the ability to co-culture and pattern different materials and cells help to enable an almost perfect part. As 3D printers are becoming more familiar, easier functions to

control the printer are easily available in terms of using of CAD (Computer Aided Design) software. Also, bioprinting help to enable rapid production of patient specific products¹⁴⁰. Generally, bioprinting processes has three dominant approaches as shown in **Figure 2-5** which are biomimicry, autonomous self-assembly and mini tissue building blocks¹⁴¹.

Figure 2-6: Different Approaches to Bioprinting: Mini Tissue Fabrication¹⁴², Biomimicry¹⁴³ and Autonomous Self-Assembly¹⁴⁴

- 1.) Biomimicry – Many technological problems has been solved using biologically inspired products ranging from aerospace¹⁴⁵ to material microstructures¹⁴⁶. In tissue manufacturing application, this can be done through replicating the cellular functional components of tissue, for example mimicking the branching pattern of blood vessels¹⁴⁷ or manufacturing physiologically accurate biomaterial type and mechanical strength¹⁴⁸. To accomplish this goal, the replication of the biological tissue must be replicated on the micro scale. This includes a sound understanding on the

microenvironment and the arrangement of the supporting and functional cell types found in the tissue and the quantity of soluble and insoluble factors found in the ECM of the biological environment-. The development of this knowledge can be obtained from fundamental research fields such as engineering, cell biology and medicine.

2.) Autonomous self-assembly – Another approach to the replicating biological tissue is to use embryonic organ as a guide¹⁴⁹. The early cellular component of a developing tissue produces their own ECM and appropriate cell signaling and autonomous organization and patterning to yield specific tissue micro architecture and function. One example of the use of such technology is the use of cellular spheroids that undergo fusion and cellular organization to mimic the developing tissue.¹⁴⁴ Autonomous self-assembly relies on the cell as a primary driver of histogenesis in directing the composition and functionalization of the properties of the tissue¹⁵⁰. This approach in printing requires a deep knowledge of the developmental mechanism of embryonic tissue genesis and organ genesis to manipulate the tissue environment to drive the embryonic mechanism in the bio printed tissue.

3.) Mini tissues – Organs and tissue comprises of many similar functional group of cells called mini-tissue¹⁵¹. These can be the smallest functional component of the tissue. Mini-tissue can be fabricated and assembled into a larger structure by either self-assembly or by design¹⁵². Self-assembling spheres of mini tissue can be used to assemble into a macro tissue using biological signals and designs; second, accurate, high resolution reproduction of a tissue unit can be designed to self-assemble into a functional macro tissue. Examples of these approach is the use of self-assembly of vascular building blocks to form a vascular branch¹⁵³. The use of bioprinting allows the accurate reproduction of functional tissue to create ‘organ in chip’ tissue model which

are maintained and connected by a microfluidic network for use in the screening of drug or cosmetics or as a tissue model for diseases¹⁵⁴.

Bioprinting requires three main components, biological living cells, bioink and a digital CAD file. Living cells – To produce transplantable bioprinted tissues, the cell component of the bioink are supposed to be autologous and patient specific. Additionally, most tissue consist of multiple cell types to perform various functions. Stem cells such as iPSCs¹⁵⁵, multipotent¹⁵⁶ and pluripotent¹⁵⁷ stem cells are regularly used as they have potentials in to repair diseased tissue and maintain normal tissue.

Bioink – To support the living cells during printing and incubation, bioinks are normally used to provide structural support and create a favourable environment for cell proliferation¹⁵⁸. Ideally, the bioink would be able to be printed accurately and maintain their structure after polymerisation, biocompatible and able to undergo controlled degradation while allowing cells to deposit ECM onto their surface. Generally, most naturally occurring hydrogels consist of either polysaccharides or polypeptides chains or a combination of both¹⁵⁹. The ink can be polymerised by physical or chemical processes which would affect the degradation and stability of the hydrogel structure. Recently scaffold free bioinks, are relatively common with researchers using high concentration of cells. The cells secrete a layer of ECM which facilitates the cell-cell communication and maturation. This approach is relatively tedious due to the high number of cells required and could easily clogged the nozzles if premature fusion has occurred. Also, final tissue size is relatively smaller. Hypoxia are also relatively common due to the lack of space.

2.5 Research methodology

Earlier in this chapter, the existing limitations of current tissue-engineered retina tissue models have been critically reviewed. One of the major limitations of existing research is the lack of a complete development of a three-dimensional model of the retina (RPE, Photoreceptors and the choriocapillaris). Most of the prior bioprinting studies focus mainly on the use of RPE cells to fabricate functional and biomimetic retina constructs. Although RPE tissue is important in the tissue therapy for retina related diseases, the interaction of choriocapillaris and the RPE through the membrane would provide a better representation of the effects of retina diseases on the membrane and the RPE tissue especially for AMD.

Till date, there is no fabrication technique that can facilitate a complete assembly of a retina. Bioprinting could improve the selective deposition of cells and hydrogel but could not produce the resolution to create an ultrathin membrane due to the limitations of the current machine's resolution and precision. Traditional casting methods could only produce a thin and uniform membrane but could not control the fabrication of scaffolds which would result in non-uniform distribution of cells in a scaffold. Thus a combinatory use of conventional casting techniques with bioprinting are used to facilitate the fabrication of the tissue model. The use of casting technique helps to produce the ultra-thin membrane to mimic the Bruch's Membrane, while precision deposition via bioprinting was used to deposit cells and biomaterials at accurate position.

2.5.1. Developing a novel ultra-thin membrane to mimic Bruch's Membrane

In this project, a suitable membrane is developed for the deposition of RPE cells. The major issues with membrane fabrication is the ability to fabricate a membrane with similar

properties to a Bruch's Membrane. Hence, an in-depth understanding of solvent casting and a new technique of fabricating the membrane is important to achieve the desired outcome.

2.5.2 Development of an interconnected 3D choroidal capillaries vasculature

3D microenvironment of a blood capillary vasculature is highly complex 3D architecture. The choroidal capillaries serve as a critical component of the circulatory system for the retina, distributing nutrient and signalling molecules. An understanding on the underlying effect of choroidal vasculature could provide us a better understanding on how neovascularisation occur at the Bruch's Membrane. However, fabrication of such intricate capillaries are complicated and prior studies not been reported to be successful to control the fabrication of the capillaries. As a tissue model, it is crucial to mimic the properties of the extracellular matrix. The properties of the ECM has to provide essential structural, mechanical and composition cues to influence the cell functions. Hence, the first part of the study aims to develop a new process of bioprinting that could print gels with similar mechanical and composition as those found in the natural ECM. Biocompatibility of the material and composition was tested to ensure that the materials used and their composition do not pose any harm on the cells of interest. Next the process was optimised further such that the base hydrogel composed of major component of the ECM found in choroid, collagen. The use of such a process, enable accurate printing of collagen with 100 μm accuracy. The experimental result has indicated that the endothelial cells are functional and vascularised in these circumstances.

2.5.3 Proof of concept: Fabrication of a complete retina tissue model

As a proof of concept shown in **Figure 2-7** below, the 3D retina tissue model was fabricated in two-steps process. The studies from the earlier chapters provided essential knowledge

towards the combinatory use of bioprinting and casting technique. The bioprinting strategy examines the possibilities of patterning on the membrane using Y-79 cells on top of a membrane cultured with a monolayer of RPE cells to mimic the BM. In-depth analysis was done on the effect of using the support material on the Y-79 and ARPE-19 cells.

Figure 2-7: Research Methodology for fabrication of retina-choroid tissue model

Chapter 3 Fabrication of Ultrathin, Free-Standing and Porous Polymer Membranes for Retinal Tissue Engineering

Synthetic scaffolds are being explored to mimic the functions of the Bruch's membrane, an extracellular matrix that acts as a molecular sieve, to maintain the metabolic exchange between the vasculature and outer retina. This work aims at fabricating an ultrathin and porous membrane, which mimics the Bruch's membrane, using a novel method. We have developed a fast, easy and single-step method to create a free-standing, porous and ultra-thin PCL membrane, through drop casting polymer blend on the liquid interface. The free-standing scaffold with the nanometre pores is investigated for the human retinal pigmented epithelial (RPE) cell response. Results demonstrate that the synthesized membrane can act as a potential prosthetic Bruch's membrane for the RPE transplantation.

3.1 Introduction

Retinal tissue engineering has been developed to regenerate the damaged host retinal cells that have been impaired by age-related macular diseases. An important component of the retina tissue construct is an ultrathin membrane that acts as a tissue scaffold for the cells; while providing sufficient mechanical strength. It is postulated that using a thinner membrane will provide improved degradation rate due to the increased surface degradation effect.¹⁶⁰ Moreover, the use of a porous membrane will be able to mimic the Bruch's membrane characteristics for efficient fluid transport^{25 161} and subsequently triggers a response to mechanical stimulation¹⁶².

Many groups have studied the fabrication of artificial scaffolds particularly synthetic membranes for retinal tissue engineering using various combination of cells and biochemical materials¹⁰⁰. Compared to naturally derived materials, synthetic materials tend to provide better mechanical strength and elasticity, thus making them a good option for producing robust and ultrathin films^{163 164}. Polycaprolactone (PCL) has emerged as a star material for fabricating the artificial scaffolds, as it exhibits slower degradation rate, and produces pH neutral and non-inflammatory by-products¹⁶⁵. PCL is also easy to process and can be fabricated into various shapes such as fibers, scaffolds and membranes^{166 167}.

Porous and permeable membranes are preferred for retinal tissue engineering, as these membranes allow the exchange of nutrients across the scaffold. Much work has been reported in literature on fabricating of porous PCL membranes^{78 168-172}. However, these synthesized membranes were lacking in mimicking the key characteristics of a Bruch's membrane, where the membrane is free-standing, ultrathin and porous in nature. Most studies have reported larger pores or macropores and thicker films¹⁷³⁻¹⁷⁵. However, in order to achieve a controlled diffusion rate which is similar to the natural Bruch's membrane, the pores need to be in the nanometer range.¹⁷⁶ Although ultrathin membrane has been researched, most has to be supported and could not be separated without damaging the membrane¹⁷⁷.

This work demonstrated the development of an ultrathin supporting membrane scaffold with favourable topography to replicate Bruch's membrane for tissue

engineered retinal pigment epithelial (RPE) transplantation. To the best of our knowledge, this is the first report where a nanoporous, free-standing and ultrathin scaffold was fabricated using a novel method. The new synthesis method was developed and presented to achieve the desired characteristics of an artificial Bruch's membrane.

The synthesis method uses the phase separation technique to fabricate an ultrathin membrane by manipulating the dispersion of the binary polymer mixture on a wet surface. The schematic in **Figure 3-1** depicts step-by-step fabrication process to fabricate the PCL membrane. The synthesised membrane had a thickness of approximately 9 μm (**Figure 3-2a**), which was formed by drop casting the Polycaprolactone/Polyethylene glycol (PCL/PEG blend) mixture at room temperature onto the surface of the PEG solution. A porous structure was formed via the demixing of the two polymers through phase separation. To

Figure 3-1: Schematic diagram illustrating the fabrication process of the ultrathin and porous PCL membrane

regulate the phase separation and thus ensure uniformity of the polymer blend composition, the solution was thoroughly mixed before dispensing. The inset of **Figure 3-2a** shows the high-speed image of the membrane forming a characteristic ring on the membrane.

During solidification of the polymer solution, a network of physically crosslinked polymer (PCL) traps and embedded solvent (chloroform) reorganising under the applied stress as the solvent dissipates.¹⁷⁸ Although the ratio of PEG and PCL is fixed by mass, there are loss of PEG into the solution due to PEG dissolution into water.

3.2 Materials and methods

Materials Preparation

Polycaprolactone, PCL powder was purchased from Perstorp (M_w 50000), with an average particle size of 500 μm and density of 1.1 g.cm^{-3} . Chloroform and Polyethylene glycol, PEG (Fluka 88276) (M_w 3350) were purchased from Sigma-Aldrich.

Membrane fabrication

To fabricate the membrane with properties similar to those in Bruch's membrane, a new method was conceptualized. In this method, 3 g PCL and 3 g PEG were dissolved in 14 ml chloroform forming a homogenous solution. 10 μl of this solution was then deposited onto a petri dish containing 20% w/v PEG- H_2O solution at 25 $^\circ\text{C}$. Due to the surface tension, PCL solution floated, allowing the membrane to be free-standing. When the solution dried after 1 min, a

porous membrane was formed in the petri dish. This happened due to leaching out of the PEG. Comparing the two polymers PCL and PEG, PCL is highly hydrophobic and longer molecular chain. These characteristics enable PCL to form a stronger bond with the solvent(chloroform). As such this slows down the evaporation rate of the solvent in this region as compared to the porogen rich regions. This forms two regions, a porogen-rich(PEG) region and a polymer-rich(PCL) region. Porogen-rich regions of the membrane can be leached with deionised water, forming a porous membrane.

The method presented combines the techniques used in dry casting, leaching and thermally induced phase separation. The polymer powder was subjected to heating at 100 °C to form a heat pressed PCL film. A clean glass slide was placed on top of the slurry before the membrane cooled down.

Membrane characterization

Figure 3-2: a) Membrane formation captured using a high-speed camera and thickness measurement using confocal image profiler b) Average water contact angle and c) Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR) scan for the fabricated PCL membrane and film

The thickness of the membrane was measured using a Plμ confocal image profiler equipped with a 100x EPI objective detection lens. The optical image

profiler utilized the z-scan of the 40 μm at a 200 nm step size for measurement. Scanning electron microscope (SEM) was employed to determine the surface morphology and porosity of the membrane. Jeol JSM-5600LV model was used for characterization, and about 9 images were captured. ImageJ software was used to analyse the pore size distribution. Contact angle measurement was done to determine the change in wettability of the membrane using an Attension theta optical tensiometer and accompanying Biolin scientific software. 10 μl Deionized (DI) water droplets were dispensed onto the 5 samples of each type to determine the contact angle. Attenuated total reflectance - Fourier transform infrared spectroscopy (ATR-FTIR), Nicolet 6700 (UK) was used to confirm the chemical nature of the synthesised membranes. The samples were dried before ATR-FTIR was performed. Atomic force microscopy (AFM) was also carried out on Asylum Research MFP3D system to study the surface roughness.

Mechanical testing

A dynamic mechanical analyzer (DMA Q800, TA Instrument) was used for the mechanical characterisation of the membrane. The procedure to quantify the tensile strength of the membrane was done in a similar fashion as reported by several researchers working on ultrathin membrane structures¹⁷⁹. The membrane was clamped on the tension clamp such that the specimen length is 5mm. The temperature was held isothermally at 25°C. The experiment ran at a strain rate of 0.5%/min with a preload of 0.001N until the membrane extended more than 150% of its original length. The specimens' widths were measured using a micrometre screw gauge.

Assessment of diffusion across the membrane experiment

Diffusion experiment was carried out on the PCL and PET membrane using a cell insert. Commercial cell insert's membrane was removed and the PCL membrane was attached onto it. PET membrane was chosen as the membrane was used extensively in RPE cells research as a viable membrane to simulate an in-vivo environment for RPE cells.¹⁸⁰⁻¹⁸² The membrane area was 0.33 cm². 300 µl of 100 µg.ml⁻¹ of 4 kDa FITC-Dextran (Sigma) was added to the insert, while 1 ml of DI water was added to the 24-well plate. The experiment was conducted at RTP for 3 h. Fluorescence reading was conducted on a microplate reader (Spark 10M, Tecan), using the default settings of 490 nm for excitation and 525 nm for emission.

Cell culture

The human retinal pigmented epithelial cell line, ARPE-19 was obtained from the American type culture collection (CRL-2302; ATCC, Rockville, MD). The cells were cultured in the DMEM:F12 medium (30-2006, ATCC, Rockville, MD), supplemented with 10% fetal bovine serum and 1% antibiotics. The cells were cultured at 37 °C with 5% CO₂, routinely trypsinized after confluency, counted, and seeded. The membrane was sterilized by soaking in 70% ethanol for 1 h and undergoing UV for 20 min prior to seeding. A cell suspension 8000 cells.cm⁻² was added on top of each membrane. After 0.5 h, 1 ml of the culture medium was added to cover the scaffold.

Cell viability

The MTT [3-(4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) assay was used as an index of the cell viability. The cells growth was observed on 1, 3 and 15 days after cultivation. ARPE-19 cells were incubated in the medium containing 100 μ l MTT (50 mg.ml⁻¹) at 37 °C for 4 h. A violet crystal was generated by the cells indicating their metabolism activity. Thereafter dimethyl sulfoxide (DMSO) solution was added (1 ml per well) until the crystal dissolved completely. After 0.5 h, the MTT solution was aspirated and optical densities of the supernatant were read at 540 nm using a microplate reader (Tecan Spark 10 m).

Live/Dead assay

To determine the viability of the cells on the membrane, the samples were subjected to the live/dead assay (L3224, Life Technologies). The samples were incubated in 2 mM calcein-AM and 4 mM ethidium homodimer (EthD-1) for 20 min in a controlled atmosphere (37 °C, 5% CO₂). The observations were done using Carl Zeiss inverted Axiovert.A1 microscope equipped with a digital camera.

Scanning Electron Microscope (SEM)

To determine the cell morphology on the membrane, the membrane was fixed using 2.5% glutaraldehyde (G7526, Sigma-Aldrich) for 10 min. The cells were gradually dehydrated using the ethanol solution and finally sputtered using a gold target. The cells were visualized under FESEM, JEOL 7600. Some of the images were false coloured to illustrate the contrast between the cells and the membrane.

Immunohistochemistry

All the antibodies and reagents used were purchased from Thermo Fisher and Sigma-Aldrich, unless otherwise stated. Cells were fixed for 15 min in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 15 min. After washing, the samples were incubated for 2 h in a blocking buffer containing 3% (w/v) bovine serum albumin in the PBS. Samples were then incubated with ZO-1 / TJP1 Antibody, Alexa Fluor® 488 conjugate (ZO1-1A12) for another 2 h. 10 $\mu\text{L} \cdot \text{mL}^{-1}$ 4',6-diamidino-2-phenylindole (DAPI) was also added and incubated for 1 h and then imaged using an Axio Vert.A1 (Carl Zeiss). Cell size was later evaluated by manually measuring the longest radius across the cell on the images using ImageJ software.

Statistical analysis

The statistical significance was determined by a Student t-test study for two groups of data or analysis of variance. P-values were presented as statistically significant and highly significant at 95% level of confidence as * $P < 0.05$, ** $P < 0.05$ is for significantly different from the rest.

3.3 Results and discussion

Evaluation of surface morphology of the membrane

The ratio of PCL to PEG was varied to study the effect on the membrane morphology. SEM was employed to analyze the membrane architecture. As can be seen in **Figure 3-3a-c**, all the samples were porous. At PCL to PEG ratio of 1:2 (**Figure 3-3a**), the additional porogen created instability during the pore formation. This resulted in the pores of various sizes, ranging from 50-500 nm.

It is also noted that the pore formation is not uniform for PCL:PEG ratio of 1:2. When the PCL concentration was increased (PCL: PEG= 2:1), a four-fold increase in the number of bandlines containing smaller pores was observed on the surface of the membrane (**Figure 3-3b and Figure 3-4**). A similar phenomenon has happened in the case of phase separation during spin casting.¹⁸³ The author suggested that the bandlines are created due to convection due to the convection rolls which are caused by the buoyancy and thermocapillary effects when a temperature gradient exist across the fluid layer. According to the paper, PCL concentration enhanced the overall evaporation rate of the solvent, resulting in the increased bandlines. The pore size distribution in the bandlines was smaller and in the range of 50-120 nm. However, if the ratio of PEG and PCL is 1:1, more uniform pore channels were observed throughout the membrane (**Figure 3-3c**). The pore size distribution was also more homogenous and in the range of 100-200 nm. The PCL membranes were translucent in nature and homogeneous as shown in **Figure 3-3d and 3-3e**.

$$Pr(d_1) = \left\{ 1 - \left(\frac{S'_2}{S_2} \right)^3 \right\} (1 - L_A) + L_A \quad \text{Eq (3.1)}$$

Where, $Pr(d_1)$ is the the porosity of the membrane, S'_2 is the radius of the dry polymer(PCL), S_2 is the radius of the wet polymer(PCL) and L_A is the apparent volume fraction of the porogen (PEG).

Based on an equation **Eq 3.1** derived from a mathematical molecular model¹⁸⁴, the porosity of the membrane can be manipulated by the type of polymer used, by varying the hydrophobic and hydrophilic regions on the chain and the length

of the where it will affect the ratio between S'_2 (radius of dry polymer particle) and S_2 (radius of wet polymer particle). However, unlike traditional solvent casting, the volume fraction of PEG changes depending on the formation rate and the dissolution rate of PEG. This is especially difficult to predict as PEG is a polymer.

Dissolution of a polymer process takes 3 steps: i.e. solvation, unfolding and swelling.¹⁸⁵ During the process, PEG will swell appreciably increasing the viscosity of the solution, restricting the mobility of the molecules and increasing the intermolecular friction.¹⁸⁶ As such, the prediction of the exact volume fraction at specific time point would require further research in terms of simulation.

$$r_{peak} = \left[\left\{ 1 - \left(\frac{v_{p(2)} d_{PL}}{d'_p} \right)^{\frac{1}{3}} \right\} + \frac{1}{\sqrt{2 \ln \left(\frac{\bar{x}}{\bar{x}-1} \right)}} \right] S_2 \quad \text{Eq (3.2)}$$

Where, r_{peak} is the pore radius of the peak of distribution of pore radius, $\bar{x}$ is the average number of porogen (PEG) in a given pore, S_2 is the radius of the wet polymer(PCL), d'_p is the density of the polymer constructing the membrane, d_{PL} is the density of the polymer after membrane formation the density of the dried polymer.

Based on Eq 3.2, the pore size of on the membrane is determined by the volume fraction of the polymer ($v_{p(2)}$), density of the polymer constructing the membrane and ratio between the density of the polymer after membrane

formation and the density of the dried polymer and the radius of the wet polymer (PCL).

Thickness of the membrane can be manipulated via either changing the concentration and different in substrate surface tension. However, these changes would affect the evaporation rate of the solvent which would affect the pore formation on the membrane. Also, creating a thicker membrane might form a non-porous film in the process as the porogen (PEG) might not be interconnected to the surface.

Extensive research interest in Bruch's membrane over the last decade had revealed specific information on the dimension and properties required for the retinal tissue engineering. Studies have shown that the pore size Bruch's membrane falls within the range of 150-210 nm¹⁸⁷. Moreover, pores or pits smaller than 150 nm are not desirable as the adhesion with the contact surface would be obstructed due to the reduced number of focal contacts between the cells and the membrane.¹⁸⁸ Hence for PEG and PCL ratio of 1:1, the synthesised membrane had the ideal pore size and would be used for further characterisations.

Effects of synthesis method on surface wettability

The surface wettability of the scaffold is a critical parameter for cell adhesion. For this reason, water contact angle of the synthesized membrane was measured and found to be 62°. It is speculated that the hydrophilic nature of the synthesized PCL membrane is due to the hydrophilic property of the PEG (**Figure 3-2b**). The fabricated PCL membrane, however, shows similar wettability

as commercial Polyethylene terephthalate (PET) membrane, which is commonly used as a cell culture insert.

Chemical composition of membrane compound

Fourier transform infrared spectroscopy (FTIR) was used to confirm the chemical composition of the synthesized membrane and to check the presence of the residual PEG after leaching. The attenuated total reflectance (ATR) mode was used to carry out the infrared spectroscopy. All the characteristics groups associated with the PCL material were identified as i) $-(CH_2)_4$ skeletal group in the $3000-2850\text{ cm}^{-1}$ region, ii) $C=O$ bond around the region 1750 cm^{-1} , and iii) $C-O$ group in $1150-1250\text{ cm}^{-1}$ region. The strong absorption for both the film and membrane were centred around 1750 cm^{-1} , indicating the presence of an aliphatic ester. The other peaks were assigned to skeletal $-C-C$ vibrations, with

Figure 3-3: SEM images of fabricated PCL membrane using different PCL: PEG ratio (a) 1:2, (b) 2:1 and (c) 1:1. Insert shows the respective magnified SEM images with a scale bar of 200 nm. Picture of the PCL membrane on a (d) glass slide and (e) cell insert. (f) Concentration curve of Dextran-FITC in water. Images illustrating the diffusion process across membrane using coloured N719 dye (g) on paper and (h) in 70% ethanol. The diffusion time span was close to 30 minutes.

a small contribution coming from $-O-H$. A slight peak around $3000-3500\text{ cm}^{-1}$

pointed to the weak presence of the hydroxyl groups, which could be due to the moisture trapped in the porous structure of the synthesized membrane (**Figure 3-2c**). No chloroform was present in the as-synthesized PCL membrane as indicated by the absence of C-Cl bond.

Figure 3-4: SEM images of bandlines on PCL membrane fabricated using PCL: PEG ratio of 2:1.

Evaluation of surface roughness of the membrane

Surface roughness of the membrane was investigated using atomic force microscopy (AFM). At least four image scans from each sample were taken to analyze the parameters. Surface roughness was evaluated as root-mean-square (R_{RMS}) value. The roughness of the synthesised films was approximately similar to the electrospun PCL membranes¹⁸⁹ with a value of 215.00 ± 0.04 nm.

$$R_{skew} = \frac{1}{mnR_{rms}^3} \sum_{k=0}^{m=1} \sum_{l=0}^{n=1} (Z(x_k, y_l) - \mu)^3 \quad \text{Eq (3.3)}$$

The surface skew value (R_{skew}) (Eq 3.3) is another important parameter, which measures the symmetry of the height distribution. The R_{skew} value for the synthesised membrane was approximately -1.780 ± 0.003 . The negative value pointed towards the dominance of pits and valleys on the surface of the film, thus indicating a porous surface. The R_{skew} value is calculated by comparing the mean of the heights against the median.

Determination of Young's modulus of the membrane

The young's modulus of the membrane is very important as it provides the initial biomechanical stimuli for the cells before a new tissue can be established.¹⁹⁰ It has been suggested that the mechanical strength of a membrane could influence the RPE cells to phagocytose the shed photoreceptor outer segments¹⁹¹. The Young's modulus of the membrane was measured to be 7.6 ± 0.9 MPa. This result was comparable to the mechanical strength of a Bruch's membrane with the reported Young's

Figure 3- 5: (a) Live/Dead Staining of RPE cells seeded on PET and PCL membrane over 7 and 12 days. SEM images of ARPE-19 cell mitosis (b) and cell spreading (c) on the PCL membrane observed at 3 days after seeding. SEM images of cells seeded on PCL (d and f) and PET (e and g) membrane after 12 days at x250 (d and e) and x1000 (f and g) magnification.

modulus values between 3-18 MPa¹⁹², thus demonstrating the feasibility of the fabricated membrane to be utilized in the tissue engineering of RPE.

Assessment of the diffusion across the membrane

Diffusion study was carried out to study the pore connectivity across the membrane. 4kDA FITC-dextran was introduced onto the membrane to determine the permeability of the membrane over the time. The fluorescence results were determined using a microplate reader (**Figure 3-3f**). The diffusion results provided the evidence that the diffusion across the PCL membrane (pore size of 150 nm) was about 5 times faster (average of $3.05 \mu\text{g} \cdot (\text{ml} \cdot \text{hr})^{-1}$) than the PET membrane (pore size of 400 nm) with an average of $0.62 \mu\text{g} \cdot (\text{ml} \cdot \text{hr})^{-1}$. This difference in the results was due to the large discrepancy in the porosity between both the two membranes. In addition, the difference in the thickness of the membrane also facilitates the diffusion. For illustration purposes, Ruthenium-based red dye N719 was also used to observe the diffusion process across the membrane with time. This was done using a cell insert fitted within a 24 well plate. Cell insert was kept on Kimwipes (Kimberly-Clark) and held upside down in a glass vial of ethanol. 10 μl was dropped on the membrane and set-up was left untouched for 30 min. Close to the 29th minute, the dye was seen straining the tissue and ethanol solution (**Figure 3-3g and 3-3h**).

Evaluation of the effect of the membrane on the cell viability and proliferation rate

The synthesised membrane was tested for the cell viability and cytocompatibility. For this, the number of live and dead ARPE-19 cells on the

membrane were computed and compared against those on the commercial PET membrane (**Figure 3-4a**). The cells were incubated on a 24 well cell insert for up to 12 days. An interesting phenomenon was observed where compaction occurred on the PET membrane after 12 days of culture, thus leading to cell void areas. The stiffening of the membrane in those regions was stipulated to cause the cell voids. Preliminary screening suggested that the PCL membrane had the cell compatibility similar to that of a PET membrane. As the membrane is opaque, cell morphology was visualised under the SEM. The anchorage-dependent RPE cells showed a flat and elongated morphology exhibiting a healthy cell state (**Figure 3-5e and 3-5g**). No difference was observed in the morphological behaviour of the two membranes. The cells on synthesised PCL porous membrane were as much capable of proliferation and adhesion as those on the PET membrane (**Figure 3-5d-g**). Cellular morphology representing the cell proliferation (**Figure 3-5b**) and spreading (**Figure 3-5c**) were identified on the surface of the PCL membrane after 3 days of incubation. Proliferation of the cells can be seen on the top surface of the membrane as the pores are too small for the cells to migrate across. Extracellular matrix (ECM) was observed to be produced by the cells due to the changes in the surface morphology of the membrane around the regions of the cells. The proliferation rate of ARPE-19 cells was then examined by MTT assay using the capability of the mitochondria to reduce the MTT tetrazolium reaction to MTT formazan. The cell growth rate on the membrane at day 1, 3 and 15 were shown in **Figure 3-6e**. The results indicated that the long-term culture of the ARPE-19 cells was feasible for extensive period of culture. Resilient to degradation is based on the membrane

Figure 3-6: ZO-1(Green) and DAPI (Blue) Staining of ARPE-19 cells seeded on (a) plate (b) PCL membrane and (c) PET membrane over 3 days. (d) Average cell size of ARPE-19 cells after 3 days of culture (e) MTT results of ARPE-19 cells on PCL membrane after 1,3 and 15 days of culture on PCL membrane

ability to withstand rupture over a period of cultivation. As PCL is a biodegradable polymer, the surface degradation by the cells after 15 days did not weakened the membrane structure.

Assessment of the epithelial barrier formation

The epithelial barrier formation was assessed using tight junction staining (ZO-1). ZO-1 staining of ARPE-19 3-day culture on all samples were generally weak. In all conditions, the ARPE-19 cells assumed a hexagonal morphology that was characteristic of the RPE cells. However, more intense fluorescence and continuous border can be observed in the ARPE-19 cells on the PCL membrane (**Figure 3-6b**) as compared to those found on the well plate (**Figure 3-6a**) and the transwell culture (**Figure 3-6c**), thus indicating a tight junction staining.

Using ZO-1 images, the RPE cell sizes were determined and quantified (**Figure 3-6d**). After 3 days of culture, the long mean axis between the cells grown on 3 different culture surfaces was measured. Long mean axis is defined as a measurement for the average longest length of the cell. The reduction of the cell size in the PCL membrane ($34.8 \pm 12.5 \mu\text{m}$) indicated that the cells formed a compact monolayer compared to the cells grown on the transwell culture ($47.8 \pm 16.26 \mu\text{m}$) and on the well-plate surface ($39.0 \pm 7.29 \mu\text{m}$).

3.4 Summary

This study demonstrated the fabrication of an ultrathin PCL membrane that could mimic the Bruch's membrane. This scaffold enhanced the ARPE-19 cell morphology and the barrier formation compared to the cells on the transwell membrane or on regular well plates. The ARPE-19 cells on the PCL membrane exhibited more uniform tight junctions indicating a well-developed barrier. The formation of this barrier is essential in many homeostatic functions including fluid transport and formation of a blood-retinal barrier. The development of a PCL membrane in this work could provide critical success for the future RPE transplantation.

In summary, we have developed a fast and easy method to create a free-standing, porous and ultrathin PCL membrane, through drop casting the polymer blend on the liquid interface. We found that the phase separation phenomenon occurred during the fabrication process resulted in a porous membrane similar to the Bruch's membrane. The synthesised PCL membrane was found to have regular and interconnected pores, indicating that adequate

nutrient exchange can take place across the membrane even when the media is separated from the membrane. This was a unique feature of the synthesised membrane as against the previously reported phase separated membranes. Cell viability of the membrane was established and found to be similar to that of a PET membrane. The membrane was also reported to have low cytotoxicity. Interestingly, the results from this study indicated that the porous PCL membrane enhanced the ARPE-19 phenotype compared to cells grown on transwell inserts and plate, as established from the cells developing better compaction and ZO-1 signaling. It was possible that the ultrathin characteristics together with the pits and pores of the membrane could have improved ARPE-19's cellular behaviour, thus enhancing the superiority of the membrane as a possible substrate for in-vitro culture. Work on fhRPE cells has also provided similar results in terms of the enhanced functionality of the RPE cells when the scaffold was porous¹⁹³. The functionality of the RPE cells in terms of the ability to phagocytose photoreceptor outer segments (POS) and protein secretion have not been studied on this platform. In the future, combined with collagen grafting, this work would enhance and enable the fabrication of a complete RPE tissue model or an implantable engineered tissue. Furthermore, the use of new processes such as bioprinting¹⁹⁴⁻¹⁹⁶ and 3D printing^{197 198} could help to control the reliability and high throughput production of these membranes and tissue constructs.

Chapter 4 Feasibility Study on Using Surface Interaction To Strengthen Bioprinted Hydrogel

In this study, alginate-based tubular structures of varying viscosity were being printed, vertically using multi-nozzle extrusion-based technique to assess the feasibility of using surface interaction to strengthen printed hydrogel. Surface interaction is the reaction or formation of polymer on the surface of substrate which means the reaction occurs simultaneously once the two different chemicals in contact with each other and each chemical is insoluble to one another so the reaction can only have on the surface¹⁹⁹. In this case, as the interaction of alginate and the calcium chloride appears to be interacting on the surface of the supported hydrogel for a limited depth. The reaction self-terminates when the calcium ions are unable to diffuse further into the hydrogel due to the increased hydrogel density.

Manufacturing challenges associated with the vertical printing configurations are discussed. A proposed measurable parameters to quantify the quality of printing for systematic investigation in bioprinting. This study lays a foundation for the successful fabrication of viable 3D tubular constructs.

4.1 Introduction

Hydrogel tubular structures for tissue engineering purposes has been researched extensively. These tubular structures are usually casted using a mould^{200 201}, centrifuged in tubes^{202 203} or co-axially extruded^{204 205}. Although the strength of such gels are much weaker than that of the polymers in terms of

tensile and ductility, the use of some of these gels improves the cell compatibility and have shown to reduce autoimmune responses²⁰⁶.

Tubular structures fabricated using bioprinting has been demonstrated with multiple materials in several reports. Examples of some of these materials that has been printed are hyaluronan²⁰⁷, alginate²⁰⁸ and gelatin-derived products¹³². They are usually built horizontally as the weight from the structure itself would not allow sufficient structural integrity of the base if it was to be built vertically. However, such method of producing tubular structure would cause irregularities in the diameter if the diameter is relatively large. Fabricating a tubular structure in the horizontal configuration also restricts the potential in constructing tubular branches on a 3-dimensional scale. To improve the strength of the hydrogel to sustain its own weight, cross-linking mechanism could be implemented to stabilise the macromolecule. The addition of cross-linkers before printing would cause premature cross-linking and thus yielding of an inconsistent result. The addition of cross-linkers as a secondary step of printing creates a potential gradient of cross-linker across the hydrogel surface. This change in the hydrogel could affect the hydrogel as the stabilization of the hydrogel would cause contraction on the hydrogel due to the difference in the hydrogel strength.

The main objective of this study was to investigate the feasibility of fabricating vertical tubular structure using a multi-nozzle extrusion-based technique. This method was derived based on concurrent deposition of crosslinking agent into the concentric tubular wall during additive printing. The cylindrical structure

was printed to provide an initial feasibility investigation to understand on the possibility of having an enclosed support structure to withstand the hydrostatic pressure present. A circular structure would provide a uniform test environment as the cylindrical nature of the tubular structure would experience uniform pressure throughout the structure and would provide an easier understanding to interpret the overall result. In a cylindrical structure, the hydrostatic pressure on the walls are uniformly distributed, this provided a fair study to determine the ability of the hydrogel mixture in holding a solvent(water). Also, this limits the effects of over-extrusion of the hydrogel during the initial acceleration and final deceleration of the nozzle during the printing process into a single portion of the print. This reduces the number of factors involved in the printing of the hydrogel.

Alginate was selected as the model material to demonstrate the feasibility of this versatile and simple method. This method could be extended for different hydrogels and their cross-linking agents.

4.2 Materials and Methods

Hydrogel Preparation

Sodium alginate powder (Sigma-Aldrich) was dissolved at 0.06 g/ml in PBS solution under constant stirring. Then 0.01 g/ml to 0.03 g/ml of Xanthan gum (XG) from *Xanthomonas campestris* (Sigma-Aldrich) was dissolved into the alginate solution. Then 0.022 g/ml of calcium chloride (Sigma-Aldrich) is added dropwise to the mixture. Additionally, 500 mM of Calcium chloride was prepared as an additional cross-linking solution.

Hydrogel Characterisation

Rheology of the hydrogel was characterized using a Discovery Hybrid Rheometer 2 (TA Instruments) using a 40 mm parallel plate geometry with a measurement gap of 0.5 mm and Peltier plate thermal control. After loading, the samples were conditioned by subjecting to 30 s pre-shear at 500 s^{-1} followed by a 1 min equilibrium before measurements were taken. Shear-dependent viscosity was evaluated using a stepped ramp of shear rate from $1\text{--}1000\text{ s}^{-1}$ and the process was done at $25\text{ }^{\circ}\text{C}$. In this method, the hydrogel used, contains the hydrogel as listed above without the addition of excess calcium chloride to understand the spreadability of the hydrogel. Measurements were taken at 10 points per decade.

Printing process

Tubular structure design and process was input into the bioprinter using BioCad (RegenHu). First, a circular structure of radius of 6mm was defined in the system as the extrusion route for the hydrogel. Then a secondary loop of radius of 4mm which is concentric with the hydrogel path was made for the dispensing route for calcium chloride. The hydrogel was placed into a time-based extruder while calcium chloride was placed in a microvalved controlled dispenser. The printing process was done in room temperature. **Figure 4-1** shows the printing process.

Figure 4-1: Fabricating vertical tubular structure using multi-nozzle extrusion-based technique.

The pressure of the bioprinter was set at 1.5 bar for the hydrogel and 0.5bar for calcium chloride. The parameters has been previously optimised to provide a uniform flow of the hydrogel and dispensing of the calcium chloride was calculated. Tubular constructs were printed using RegenHu's Biofactory. Hydrogel was printed through a 0.25 mm syringe needle (Needle DD-135N-N4) while the CaCl_2 solution was dispensed through a 0.3 mm needle tip. The path speed of the hydrogel was 500mm/min while the path speed of the CaCl_2 was 100 mm/min. CaCl_2 solution's path speed was much lower than the hydrogel to allow it to sufficiently fill up the tube during dispensing. The layer thickness of the hydrogel was set at 0.2 mm. To allow time for the layers to fuse before allowing the gel to cross-link, the printer was programmed to dispense CaCl_2 only after 3 layers has been built. Subsequent layers was added in the vertical

axis after gelling interaction was achieved in the first 3 layers, providing it sufficient structural integrity to support the next few layers of hydrogel. To show the feasibility of this printing strategy, the structure was printed to a height of 4.8 mm.

Image Analysis for Measurement of Printing Quality

Obtained images were processed using ImageJ (National Institute of Health, USA). The images were calibrated to correlate dimension of the physical objects to the pixel size. Image was then processed using an 8bit image and later, undergone proper thresholding. The tubular structure was measured in terms of size, area and perimeter with the image processing methods. Circularity results were calculated by comparing the difference in the ratio between the radius obtained from the perimeter and the area. When the ratio is approximate to 1, there is no distortion in the circle.

4.3 Results and Discussion

Challenges of bioprinting tubular structures

Printing tubular structures in the vertical configuration is very challenging. The strength of the base material must be robust enough to withstand the weight of the entire structure. This is especially difficult as hydrogel is a soft material with high water content. Insufficient structural strength of the hydrogel base would have resulted in the collapse of the tubular structure in vertical configuration. The viscosity of the material thus must be relatively high in order to sustain the compressive pressure resulted from the upper layers of the tubular structure. However, high shear force will be needed for extrusion of

viscous materials, which might impact the cells negatively and reduce cell viability. Moreover, from Stokes-Einstein equation, it can be predicted that the increase in viscosity would have an inverse effect on the diffusion rate of molecules ²⁰⁹. Low diffusion rate of hydrogel will inhibit nutrient exchange into the hydrogel, which is critical for cellular growth and survival. Thus, there is a need to optimize the viscosity of the printable hydrogel for printability and diffusion rate.

Optimizing the viscosity of printable hydrogel

The printable hydrogel needs to be optimized to have low viscosity during printing and sufficient mechanical strength upon printed, with good diffusion rate for nutrient exchange. One of the ways to overcome such conflicting requirements would be to complete gelation after printing. Although some research has been done on cross-linking alginate gels simultaneously during printing but the process was shown to be relatively slow as they were done in the crosslinking solution or on the surface of the alginate solution. Thus the resolution of the fabricated structure is influenced by parameters such as surface tension and the speed of the extrusion, rendering a non-direct way to control resolution.

In this study, Xanthan gum (XG) was selected to formulate an optimal viscosity for alginate to retain its shape fidelity after printing. Xanthan gum is an anionic polysaccharide produced by the bacterium *Xanthomonas* *Capestris*. Xanthan gum has been used in multiple applications ranging from food²¹⁰, agriculture²¹¹,

petroleum²¹² and pharmaceutical industry²¹³. It is used mainly as viscosity enhancer and stabilizer in blends and has been reported to contain bioadhesive properties²¹⁴. Xanthan gum is also a biocompatible and biodegradable material that has been used in multiple study as a tissue scaffold and most recently been used as a support material for bioprinting of cardiac heart patches²¹⁵. As xanthan gum is also a thickening agent, viscosity of the material can be easily tuned. Since Xanthan gum has only weak interaction with CaCl_2 , it serves as pure filler and will have minimum effect in the crosslinking process. To understand how viscous the hydrogel is and its shear thinning properties, the rheology behaviour of the hydrogel was characterized (**Figure 4-2**). With increasing amounts of Xanthan gum, the solution tends towards a more viscous behavior as expected. This viscosity will affect the amount of hydrogel extruded and the overall printing fidelity of the construct.

Figure 4-2: Viscosity as a function of shear rate for Alginate-Xanthan Gum Gel. Xanthan Gum concentration was varied from 1% (blue diamond), 1.5% (orange square), 2% (grey triangle), 2.5% (yellow cross) and 3% (blue asterisk).

Quality of Printing

Tubular constructs was biprinted successfully in the vertical configuration using the concentric printing method developed in this study (**Figure 4-3**). In general, the designed CAD profile was replicated successfully in the biprinted hydrogel. The printing quality was represented using the following measurements, namely Tubular Length (t), Wall Thickness (w), and Roundness (R). For calculation of roundness in the equation, we require the perimeter (P) and the area (A) of the inner cavity of the tube. Printing effect such as Spreading ($e1$ and $e2$) and Opaque Layer Thickness(OW) were also proposed and discussed.

Figure 4-3: Measurements parameters to quantify the printing quality of a biprinted tubular construct.

Tubular length (t) helps to quantify if the layer thickness was calibrated correctly such that sufficient hydrogel was deposited to form layers additively, and eventually achieve the desired tubular height in vertical configuration. To optimize the printing, a group of printing parameters such as extrusion speed,

nozzle diameter and pressure of the extrusion were synchronized to act as a calibrated system to deposit material accurately on top of previous printed layers²¹⁶. With proper calibration, defects and delamination between the layers should not occur. The wall thickness, w , determines the minimum resolution of material deposited onto the platform. The spreading effect, e , was calculated as an angle from the platform. Spreading displayed the effect of viscosity to the form factor of the construct. The Opaque Layer Thickness (OW), show the degree of calcium diffusion into the material. While the calcium diffused into the material, the ion crosslinks the Alginate-XG solution. This reaction also caused the structure to turn white, allowing it to be quantifiable through the thickness of the diffusion zone.

Wall thickness

The wall thickness was measured to develop a presentation of how the viscosity affects the resolution. From the results in **Figure 4-4**, it can be seen that at 2% XG concentration, the width variation is minimal; indicating that printing these material is most stable at that viscosity range. Also it can also be established that the concentration of 2% has less material spreading as compared to the 1% XG concentration.

Figure 4-4: Wall thickness (mm) vs Concentration of XG

Roundness of tubular construct

To determine printing error for hydrogel is significantly difficult as compared to hard 3d printing thermoplastic (PLA). Small deviations in the environmental factors such as humidity and temperature would have adverse effects on the printing conditions. Combined with the shape and volume factor influencing the localised evaporation and humidity on different shapes and design and the change in time for printing and the heat generated in the machine. The resultant would create a relatively complex problem. Calculation of an “error” in this way, would only provide enough evidence that the error is true if the print time, print dimension and shape remains the same.

Simple shapes such as a tubular structure was selected as initial study as this would have provided a clear evidence if more complicated shape could be produced. Thus, in simplifying the effect of the error, roundness of the object was being used to determine the printing capability of the print. Generally, there are multiple methods for measuring the roundness of an object. In this

case, roundness of the construct was determined from the ratio between the square of the perimeter and the area of the cavity (see eq 4.1). To calculate A and P in **Eq 4.1**, ImageJ was utilized to analyze the cavity. The image was thresholded and the result is calculated based on the number of pixels for area of the cavity, A, and the length of the overall circumference pixel for the perimeter of the cavity, P.

The ideal roundness of a tubular construct is 1.0, which implies that the area and the perimeter are equal. In this experiment, we showed that the pre-designed roundness was achieved with 2% XG, indicating an apparent optimal viscosity threshold exists for a desired roundness value. The printed tubular structure showed increasingly out-of-roundness when the viscosity was too low at 1% XG or too high at 3% XG. It was estimated that the movement of the printing platform may have affected the shape fidelity as the extrusion pressure and speed were kept constant for all viscosity materials. When the viscosity is too high, the volume of hydrogel that was dispensed may have been insufficient, causing the material to be unstable and out-of-round.

Figure 4-5: Circularity at different concentration of XG

$$R = \frac{(P)^2}{4\pi.A} \quad \text{Eq (4.1)}$$

Spreading

Better shape fidelity can be derived from the spreading angle of the construct. Mirroring the concept of contact angle for droplet spreadability, the right and left angles of the base layer were measured and averaged. The optimal spread angle would be 90°, indicating no spreading has occurred and thus representing the best printing quality with the material. In this work (**Figure 4-6**), best printing quality was achieved for formulation of 1.5 to 2.5%. As Xanthan gum ratio is increased above 2.5%, the spreading of the hydrogel increases as attributed to 2 factors in the hydrogel, i.e. the increased in viscosity and the weight of the hydrogel. The increased concentration of xanthan gum increases the viscosity of the hydrogel which reduces the wall thickness of the extruded

hydrogel filament. Coupled with the increased weight/volume of the hydrogel, this cause the hydrogel to form an unstable structure and in turn lead to spreading.

The decrease in the spreading angle at 3%, could indicate that the initial layer does not have sufficient surface area to provide sufficient compressive strength to withstand the weight of the hydrogel that was layered on it.

Figure 4-6: Spread angle at the different concentration of XG

Opaque layer thickness

The constructs develop an opaque interior due to the cross-linking effect of Calcium ion on the Alginate Xanthan gum gel. The opaque wall is related to the diffusion rate of the cross-linking agent into the hydrogel.

$$D = \frac{kT}{6\pi\eta r_H} \quad \text{Eq (4.2)}$$

Based on estimation from the Stokes-Einstein equation in **Eq 4.2**, where, η is the viscosity of the solvent, D is the diffusion coefficient, r_H is its hydrodynamic radius, T is the absolute temperature of the solution, and $k = 1.38 \times 10^{-23}$ J/K is

the Boltzmann constant. The diffusivity of a material is inversely proportional with viscosity. The increase in concentration of XG increases the number of molecular chains blocking the path of the ions as it diffuses into the gel. Thus the viscosity increases, reducing the diffusion rate as shown in the graph (Figure 4-7).

Figure 4-7: Opaque thickness of tubular construct at different concentrations of XG

Figure 4-8: Printed alginate-XG tubular structure. (Scale: 1cm)

The photos of the printed construct are shown in **Figure 4-8**. When the Xanthan gum used is too low (1%), we can see that there is an overall spreading of the hydrogel as compared to the rest of the construct. Additionally, in **Figure 4-8e**, the hydrogel printed showed substantial shrinkage after crosslinking. This further strengthens the need of an optimal material viscosity as crosslinking diffusion was too slow at high viscosity, thus affecting the overall shape. This can be seen from the defects and cave-in showed in **Figure 4-8j**.

The constructs developed an opaque interior due to the cross-linking effect of CaCl_2 on the Alginate Xanthan gum gel, creating a gradient of mechanical properties radially towards the outer wall of the gel wall. From the results, it could be concluded that 2% xanthan gum produced the best printing quality, with optimal roundness, minimum spreading and optimal diffusion rate.

Extending Process Capability

Demonstrating the process capability of this novel approach, a tubular construct of 15mm length was fabricated in the vertical configuration using the 2% Xanthan gum-Alginate gel as shown in **Figure 4-9**.

Figure 4-9: 15mm tall tubular construct

Compared to printing of 4.8 mm tubular structure, there seems to be a shrinkage effect caused by the CaCl_2 gelation interaction at higher tube height. This shrinkage could potentially be resolved by using a lower concentration of CaCl_2 . In summary, the tall tubular construct was relatively straight with little deviation from its designated shape, and minimum spreading.

4.4 Summary

This study has shown that tubular constructs could be successful bioprinted in vertical configuration by controlling the viscosity of the hydrogel and using a new multi-nozzle printing approach to achieve in-process crosslinking. The cross-linker was able to embed through the surface of the hydrogel, stabilising the hydrogel. Hydrogel tubular structures of large diameter with good shape fidelity and integrity were demonstrated. Limitation of this method includes shrinkage induced during crosslinking process, which require further process optimization. We also demonstrate the capability of this fabrication technique with a tall hydrogel tubular structure of at least 15mm length in vertical configuration. There is potential to further develop this concentric printing method to create a branching tubular tree structure with further fine tuning of tool path design.

Chapter 5 Development of Supported Hydrogel Printing System

5.1 Introduction

While numerous other direct methods and strategies exist to print viable cell into a structure such as the use of ink-jet, extrusion and laser-induced forward transfer (LIFT), the ability to print structures of beneficial print resolution while obtaining good cell viability is somewhat limited. The printing resolution of hydrogel could often affect the long term viability and metabolism of the cells embedded in the hydrogel²¹⁷. In one study, the effect of the spacing of the grooves affects the mass transport of media in stiff hydrogel. This in turn greatly affected the cellular metabolic and phenotypic activity of the cells. In some cases, necrotic cores can be observed in embedded hydrogels that are caused by transport limitation²¹⁸.

One of the conventional bioprinting technique is the use of an extrusion based printing technique²¹⁹. This form of printing dispenses a liquid using either a pneumatic or a mechanical pump to create a positive pressure in the syringe. Extruding the solution in a controlled manner, this form of printing has been well documented in many studies as the process is simple and the machine is inexpensive²²⁰. While the use of this process has been successful in fabricating 3D scaffolds and cell embedded hydrogel, the live cells has to undergo tremendous shearing during dispensing as the material used has to be relatively viscous²²¹. As such this process of printing would result in either lower cell

viability or lower print resolution depending on the viscosity of the material used^{222 223}.

Recently, extrusion printing in a microbead support bath has been becoming a general standard of printing to eliminate the need to design supports for the biological construct^{224 225}. This reduces the vast array of problems from biological construct interacting with the dry air and enables printing of resolution of up to 200 μm ²²⁶. However, these supporting bath are still susceptible to temperature changes where the working temperature has a variation of between $\pm 1^{\circ}\text{C}$ and would require higher pressure to dispense the solution into the bath. Depending on the height of printing, the volume of material extruded would be different due to the varying pressure difference between the nozzle of the syringe and the atmosphere.

One method of overcoming this challenge is the use of dual/multiple extrusion printing, in which two or more heads are used to dispense either scaffold hydrogel or cells embedded hydrogel onto the platform²²⁷. The use of multiple print heads is largely common in the field of bioprinting as the gel stiffness are relatively weak and would require additional support to enable the structure to be stable.

One of the supporting materials that has been used commonly is pluronic^{153 228}. Pluronic is a synthetic reverse thermoresponsive hydrogel. It has been widely used as a support material because it can be easily removed when the scaffold is immersed into cold liquid²²⁹. Since pluronic is a physically crosslinked hydrogel, it is susceptible to absorb water from the surrounding and when the

concentration of pluronic in the hydrogel is below the critical gelation concentration, the hydrogel would structure would be eroded²³⁰. As such, the interaction time between pluronic and hydrogel of interest are usually instantaneous to prevent the gels from interacting. However, this is not possible for some natural occurring hydrogel that would need to be slowly cured over a period of time such as collagen^{231 232} and fibrin²³³.

In this chapter, a new hydrogel process based on the previous chapter design will be explored. Based on the principles of surface interaction explored in chapter 4, the characteristics of the experiment was further improved to produce a new printing strategy that could encompass printing of hydrogels of low viscosities. In this method (**Figure 5-1**), the support material alginate-pluronic was printed using extrusion printing and cells embedded hydrogels was inserted and cured indirectly. The solvent interaction of the two printed materials will lead to the formation of a temporary hydrogel barrier layer that provides the necessary shape fidelity definition during printing. The interaction of both the hydrogel of interest and pluronic is self-limiting through the formation of an interfacial wall during printing. As the interfacial wall stabilises, the newly formed wall obstructs the solvent transport across the hydrogel, thereby forming a barrier to reduce diffusion of solutes across the interface during the curing process. As a result, this reduces the intermixing of the hydrogel and therefore produce a higher resolution structure. This improvement in the process would enable different types of long curing hydrogels to be printed, expanding the choice of bioinks to be utilized in

bioprinting. In chapter, the swelling and surface interaction of the hydrogel will be investigated and the effect of surface interaction will be discussed.

Figure 5-1: Experiment flow diagram of the processes involved in fabrication of the cells embedded hydrogel structure.

Another critical criteria of a functional bioprinting process is the biocompatibility of the process. The printed material, is the ability to optimally support cell development and attachment, during and after printing. The printed hydrogel creates a suitable 3D environment for cells to proliferate.²³⁴ The process of printing must not create negative effect to the cells that reside within the printed product¹²⁹. Currently, this is still a challenge as the cells extruded through a nozzle generally undergo tremendous shear stress and would have detrimental effects on the cells²³⁵. Different types of hydrogel materials should be evaluated as they will elicit different cell response with different cell types²³⁶. For example, the usage of Arg-Gly-Asp (RGD) adhesive

peptides on marrow stromal cells may block the mineralization and help promote osteogenesis of MSCs.

The biocompatibility of printed material is also a function of the processing technique. Harsh chemical such as glutaldehyde and exposure to unfavorable environment might cause both long and short-term harm to the cells. Thus, in this chapter, two different materials namely, GelMA and Collagen Type I will be used to evaluate the biocompatibility of this new process. Collagen Type I was used in this experiment as it is an ideal material to be used in Tissue Engineering, it is found in most of the cell extracellular matrix and provide an excellent environment for cell. Furthermore, it is used heavily in research and medical devices to provide better cell proliferation and would form a good comparison for future experiment¹⁶³. However, collagen is difficult to be printed as their viscosity changes as it cures over time. It has an extremely long cure time (>20 min)²³⁷. As collagen viscosity is extremely low, the shape fidelity of their printed products are generally bad with resolution in millimeter²³⁸. Even after curing, the hydrogel is generally weak and would collapse if the hydrogels are repositioned. Thus, the use of collagen would provide a good challenge to how this process could benefit the use of collagen in future bioprinting.

To further proof the biocompatibility of the process, HUVECs are used in the experiment to test the functionality of the process. Primary cells such as HUVECs are extremely sensitive to their surrounding and can quickly fall into dormancy if not treated properly²³⁹. The use of HUVECs will enable us to understand how

choroidal capillaries cells would behave in the collagen. Although endothelial phenotypes are dependent on the organ and origin, it is one of the cells that is readily available.

5.2 Materials and Methods

Fabrication of GelMA

Gelatin methacrylate (GelMA) was fabricated by reaction between methacrylate anhydride and gelatin at 50 °C in Phosphate buffer saline (PBS) similar to the method that previously described by Narbat et al²⁴⁰. The reaction was run for two to three hours under constant stirring condition in the dark. After that the reaction was stopped by diluting the solution fivefold with PBS. The diluted solution was further dialyzed with deionised water by using 12–14 kDA molecular weight cut-off (MWCO) dialysis tubes for 1 week to remove excess methylacrylate anhydride. Next, the GelMA was frozen overnight at –90 °C, lyophilized for 5–7 days until fully dried, and stored at –30 °C before further use.

Fabrication of support material and scaffold hydrogel

Support structure hydrogel was composed of 24.5% Pluronic F127 (Sigma-Aldrich) and 2% Alginate (Sigma-Aldrich) dissolved in deionised water. GelMA hydrogel was composed of 5% of the freeze-dried GelMA and 0.1 M of Calcium Chloride (CaCl₂) dissolved in deionised water, pH of the solution is subsequently tuned to 7 using 1M NaOH which was added dropwise. Collagen hydrogel composed of 2 mg/ml of high concentration Type I Collagen (Corning, 354249) which 0.1 M CaCl₂ was subsequently added to the solution.

Evaluation of surface interaction of calcium ions on pluronic structure

A square structure (**Figure 5-2**) of lengths $1 \times 1 \text{ cm}^2$, height (thickness) of 2 mm and a z layer resolution of 0.2 mm was designed in BioCAD™. The structure was printed by using a pneumatic extrusion-based bioprinter (Regenhu, Villaz-St-Pierre, Switzerland). Subsequently, 100 μl of the GelMA solution with CaCl_2 was added to simulate the interaction between the Ca^{2+} cation and the alginate in the Pluronic structure. To ensure that the thickness of the material is consistent and the test to be unbiased, a fixed concentration of Alginate and Pluronic was used while varying the calcium concentration.

Figure 5-2: Sample Design for evaluation of surface interaction on the support material.

Figure 5-3: General fabrication process for 3D hydrogel scaffolds (A) Printing of sacrificial alginate-pluronic mould (B) Dispensing of collagen/GelMA solution into the mould structure, (C) Interfacial reaction between the sacrificial hydrogel and collagen/GelMA solution, (D) Curing of hydrogel either via UV crosslinking for GelMA or thermal incubation for Collagen, (E) Removal of support by soaking the printed structure in cold PBS solution.

Printability test and printing of 3D complex structure

A grid CAD file was designed using BioCAD™ which attached to the pneumatic extrusion-based bioprinter (Regenhu, Villaz-St-Pierre, Switzerland). The hydrogel and the support structures were loaded in 5 ml syringe before printing. The printing condition of all concentrations was at stage moving speed of 500 mm/min, pressure of 5 bar, temperature at 27 ± 2 °C. 27 G nozzle was used for printing. After printing, the GelMA hydrogel samples were cured by using UV flood curing system (Techno Digm, Singapore) for 120 seconds and soaked in cold PBS for 15 min before the further experiment. For the collagen structure, the samples were cured in an incubator at 37 °C for 30 mins before being soaked in cold PBS.

Fixation for SEM

All the hydrogels need to go through drying process before using SEM to examine the structure of the GelMA specimens. Firstly, all the hydrogels were soaked into 2.5% glutaraldehyde in distilled water for 2 hours. Next, they were submerged into cleaned distilled water thrice, each for a period of 15 minutes. After that, the hydrogels were dehydrated with different concentration of ethanol as any water content in the specimens will affect the resolution of the SEM photos. The hydrogels were soaked into 25% ethanol, followed by 50%, 70% and 90% ethanol for 10 minutes. It was then soaked into 100% ethanol twice for 10 minutes. Finally, the hydrogels were dried by BAL-TEC CPD 030 Critical Point Dryer. The dried samples were then gold-sputtered using JEOL JFC-1600 auto fine coater before using JEOL, JSM-5600LV SEM for analysis.

Mechanical Testing

10mm diameter by 5mm height Disc of GelMA hydrogel was printed in a similar process as those described in **Figure 5-1**. Before the compression testing, the hydrogels were soaked in 1X PBS solution for 1 hour. The hydrogels were blotted lightly with a KimWipe and tested at a rate of 20% strain/min using an Instron 5566 mechanical tester. The data was plotted using an excel sheet and the compressive modulus was determined as the slope of the best fit line at the initial region of the stress-strain curve.

DAPI/F-actin staining

HUVECs (Human Umbilical Vein Endothelium primary cells, Lonza) with a passage number of 5-6 were cultivated in endothelial growth Bullet Kit (EGM-2, Lonza) supplemented with 1% antibiotic/antimycotic solution (PAA, GE Healthcare). Culture medium was replaced every 2-3 days and cells were grown at 37 °C in the presence of 5% CO₂. After the cells reached more than 80% influences, they were subcultured by using trypsin/EDTA (CC-5012, Lonza) and mixed with the 2 mg/ml collagen at a cell density of 3×10^6 cells/ml. The bioprinted samples were plated on a 24-well plate and soaked in cell culture medium for 30 min to remove the sacrificial materials. After that, they were stained by ActinGreen™ 488 ReadyProbes® (Life technologies, Thermo Fisher) and NucBlue® Live ReadyProbes® (Life technologies, Thermo Fisher) reagents and observed under inverted microscope (Axio Vert.A1, Carl Zeiss).

Immunofluorescence

HUVECs were cultivated as described above. For immunofluorescence, the cell-laden hydrogel samples were plated into 24 well plate up until day 7. In order to investigate the cell differentiation of HUVECs, CD31 and von Willebrand Factor (VWF) expression were selected. The samples were rinsed in DPBS a few times and fixed in 4% formaldehyde solution (Sigma-Aldrich, USA) in Dulbecco's Phosphate Buffered Saline (DPBS) (Hyclone, GE life science) for 30 minutes. After that the samples were soaked in blocking solution (5 wt % BSA, 0.5 wt % Tween 20 in DPBS) for 2 hours at room temperature. Subsequently, the cell membranes were permeabilized in 0.25%(v/v) Triton X-100 (Bio-Rad, USA) in blocking solution for 20 minutes and washed with DPBS for three times. The samples were soaked in the primary antibody staining with 1/100 dilution of mouse monoclonal anti-CD31 antibody (Life technologies, Thermo Fisher) and 3 µg/ml of VWF mouse monoclonal antibody (Life technologies, Thermo Fisher) in DPBS overnight at 4 °C. The samples were washed with blocking solution three times with 5 minutes intervals between the washing steps. After primary antibody staining, the samples were and 1/500 dilution of Alexa Fluor-555 conjugated goat antimouse secondary antibodies (Life technologies, Thermo Fisher) in DPBS for 2.5 hours at ambient condition. Subsequently, the samples were washed in blocking solution three times with 15 minutes intervals between the washing steps, followed by a drop of NucBlue® (Life technologies, Thermo Fisher) staining for 20 min. After rinsing, fluorescent images were taken by using florescent microscope (Axio Vert.A1, Carl Zeiss,).

Haematoxylin and Eosin (H&E) staining

The samples were fixed with formaldehyde (10% v/v) overnight followed by dehydrated with ethanol by a series of concentrations (v/v): 70%, 80%, 95% (twice), and 100% (three times). Samples were soaked in each ethanol concentration for 45 minutes. Later, samples were soaked in xylene for three times with 45 minutes interval between each time. Subsequently, the samples were embedded in paraffin and 7 µm sections were cut by using a microtome (Leica, Germany). The sections were immersed in water bath at 37 °C and mounted on Polysine® adhesive coated glass slides for better adhesion. The slides were left to dry overnight at 37 °C. The sections were rehydrated in xylene, then a decreasing series of alcohol concentrations: 100%, 95% and 70%, followed by immersing in water. Conventional haematoxylin and eosin (H&E, Sigma, USA) staining was performed and the samples were dried for a few hours. Slides were examined under light microscopy.

Statistical analysis

The statistical significance was determined by a Student t-test study for two groups of data or analysis of variance. P-values were presented as statistically significant and highly significant at 95% level of confidence as *P < 0.05, **P < 0.05 is for significantly different from the rest.

5.3 Results and discussion

Evaluation of surface interaction of calcium ions in alginate-pluronic support structure

The stability of the support material after interaction with calcium was evaluated to confirm and optimise the competency of using Alginate-Pluronic as a support structure. As shown in **Figure 5-4**, there was minimal change in the dimension to the dimension when the calcium concentration is between 50-100 mM for more than 2.5 min. The calcium ions resulted in slight swelling due to the formation of Calcium-Alginate interaction in the Pluronic hydrogel [21]. If excessive amounts of calcium ions was used, the structure would expand uncontrollably. This is possibly due to the calcium ions displacing the stable micelle structure and reducing the interaction between the pluronic chains, thus causing an un-stabilisation of the pluronic structure. If pure pluronic was used in the reaction, the solvent (water) will interact and lower the concentration of pluronic on the surface of the hydrogel-solvent interface, resulting in an erosion of the pluronic structure. From this result it can be concluded that the use of an alginate pluronic structure with an optimised calcium concentration would result in a structurally stable support structure that could delaying and reducing solvent interaction.

Figure 5-4: (A) Comparison of the effects of the type and concentration of support structure changes over time. (B) Microscopy evidence on the effect of using pluronic as support as compared to pluronic alginate, using Calcium-GelMA printing material. Scale bar: 500 μ m.

Solvation of pluronic support structure

Electrostatic interaction play an important role in the structural properties of hydrogels. Slight changes in the pH, solute concentrations and their temperature could result in changes in their physical properties. This is due to the interaction of the polar and ionic molecules interacting with the polar solvent(water). The hydrogel's range of deformation is dependent on the presence of the interacting groups and their distribution on the molecule.

Figure 5-5: Comparison of Interaction of solvent on Pluronic/Alginate support(A) and Pluronic(B) at hydrogel-water interface. Due to the lack of calcium ions to stabilise the structure, water molecules could easily destabilise pluronic micelle formation which leads to weaken supports.

The interaction between pluronic and water can be described in **Figure 5-5A**.

Initially, pluronic gel acts as its own a membrane against the solvent. A hydrostatic pressure will exist between the interior of the gel and the water phase. Thus, a pressure gradient exists through the interface. This presence of hydrostatic pressure is influenced from the swelling pressure that is exerting on the gel to prevent it from swelling. This rise in hydrostatic pressure increases

the chemical potential of the water in the hydrogel to match that of the solvent outside the membrane. However, for pure pluronic, this rise of solvent concentration in the hydrogel causes a non-volume preserving deformation on the gel. Due to the weaker H-bond interaction between pluronic molecules, the increased in water molecules competes and displaces the pluronic molecules. This disrupts the micelle structure and reduces the pluronic hydrogel back to its solution state. This interaction will happen until the solvent has almost similar pluronic concentration as the pluronic critical micelle concentration.

The addition of alginate as described in **Figure 5-5B**, provides a better approach to produce a support structure. In this system, calcium ions are adsorbed onto the surface of the surface of the alginate pluronic hydrogel. The calcium displaces the sodium ions on the alginate chains thereby forming an ionic crosslink within the hydrogel. Over time, as more of the alginate interacted, the gel interaction would be increasingly stabilised and produces a robust membrane in the hydrogel. The formation of calcium-alginate helps to facilitate a reduction in the pluronic-solvent interaction by forming a stronger ionic interaction with neighbouring alginate and reduce the erosion of pluronic into the solvent. Thus, causing intermixing of the support and base material.

Figure 5-6: Top Surface of base hydrogel, (A) Calcium-GelMA supported by Alginate-Pluronic, (B) Calcium-GelMA supported by Pluronic, (C) Calcium GelMA casted, (D) GelMA Supported by Alginate-pluronic, (E) GelMA supported by Pluronic, (F) GelMA casted.

Effects of support hydrogel on base hydrogel

The interaction between support hydrogel would have a definitive effect on the base hydrogel. As both supporting and base hydrogel has a tendency to compete for water due to their hydrophilic nature, this can be seen from the differences in **Figure 5-6D, E and F**. Due to higher hydrophilic molecules from the addition of alginate in **Figure 5-6D**, it was observed that a higher shrinkage of the GelMA skin compared to those which are printed in Pluronic and those casted. This absorption of water is evident from the swelling as observed in **Figure 5-4**. The reduction of water content would increase the salt concentration within the hydrogel which could cause crenation and eventually cell death.

Precipitates can be observed in **Figure 5-6A, B and C**. This is due to the addition of calcium into the hydrogel mixture before curing. Evidently, calcium precipitates are not as noticeable in the Calcium-GelMA that was printed with

alginate pluronic hydrogel. This difference in precipitation could be caused by the interaction of alginate and calcium at the interface. The Calcium-GelMA casted has a higher precipitation as compared to those printed in the pluronic. This reduction is possibly caused by general diffusion of calcium across the hydrogel during the curing process.

Remarkably, the shrinkage in Calcium-GelMA printed in alginate hydrogel is not as severe as those in GelMA as the shrinkage lines are not present on the Calcium-GelMA. This is possibly due to the increased in salt content that help to reduce the difference in hydrophilicity between the two hydrogels. The formation of an alginate wall has also helped to reduce the shrinkage effect further.

The visible lines denoting the different layers was not as apparent on the hydrogel supported by Pluronic support as those found on the Alginate-Pluronic support (**Figure 5-7**). On the Alginate-Pluronic supported GelMA, the interaction of calcium on the interface prevented the supports from being eroded, thus printed layers attributed from the bioprinting of the supports was very obvious.

Figure 5-7: Comparison of Calcium-GelMA printed in (A) Alginate-Pluronic support and (B) pluronic support.(C) A Printed GelMA disc supported by Pluronic hydrogel after curing

Compression properties of base hydrogel after interaction

Figure 5-8: Compression modulus of (A) Calcium-GelMA supported by Alginate-Pluronic, (B) Calcium-GelMA supported by Pluronic, (C) Calcium GelMA casted, (D) GelMA Supported by Alginate-pluronic, (E) GelMA supported by Pluronic, (F) GelMA casted.

The compression properties of the hydrogel analyses how the interaction of the ions and the support materials have on the base hydrogel as shown in **Figure 5-8**. The addition of calcium into the GelMA reduces the compressive strength of the hydrogel. This is possibly due to the calcium ions interaction with the crosslinking sites. This reduces the number of crosslinks on the GelMA and

reduces the overall tensile strength of the base hydrogel.²⁴¹ Additionally, the calcium ions acts as a salt to deteriorate the cohesion of the triple helix structure of the GelMA resulting in a comparatively “free” structure.

Compared to the GelMA supported with pure Pluronic, a future reduction in the GelMA mechanical properties can be observed, this reduction is possibly due to the interaction of Pluronic on GelMA solution during the curing process. As previously mentioned, the Pluronic molecule mix readily with the GelMA solution in the absence of a calcium alginate wall. This mixing of the GelMA could reduce the GelMA concentration and introduce additional defect sites on the hydrogel.

The use of Pluronic-Alginate support showed an increased in the GelMA compressive modulus. This increase in compressive modulus could be due to the reduction of divalent ions in the base hydrogel solution due to the interaction of alginate at the interface. This helps to increase the cohesion of the triple helix structure of the GelMA. It is also possible that this increase could be due to residues of the calcium alginate in the base hydrogel, therefore providing an exceptionally higher compression modulus.

***In vitro* evaluation of hydrogel process**

L929 fibroblast cells have been frequently used for preliminary biocompatibility test²⁴². To enable the optimal survivability of cells embedded in the hydrogel, the pH of the printing solution was tuned to approximately 7 and the osmolarity of the solution to be between 200-300 mMol which is the physiological comparison. In this experiment, L929 was mixed with 5% Ca-GelMA solution. As

shown in the Prestoblu result and the live-dead results in **Figure 5-9A**, it can be concluded that the alginate-pluronic support structure do not have any effect on affecting the cell viability of the cells in the hydrogel. The increasing trend from the Prestoblu, demonstrated that the cells are proliferating at an accumulative rate, similar to the results from the TCP culture. The initial cell death could be resulted from use of the photo-initiator in the hydrogel²⁴³. The actin staining in **Figure 5-9B** and SEM images showed that the cells have migrated to the surface and adhered and spread relatively well.

Compared to a traditionally casted hydrogel, this process of printing enables higher density of cells to be viable due to the increased surface area to volume of the hydrogel^{244 245}. This reduce the risk of necrosis which is present most casted hydrogel due to the lack of nutrient transport especially for long cultures (>20days) and in hydrogels²⁴⁶.

Figure 5-9: (A) Comparison of Live/Dead Assay on L929 over 7 day. (B) F-actin staining of L929 after 14 days (C) Metabolic activity of L929s In GelMA from day 2-6 (n=3)

Figure 5-10: SEM Images of L929 (Day 14) on GelMA, at different magnifications: (A)x100 (B) x500 (C)x500 and (D)x2000.

To evaluate if this process can be used for angiogenesis study, HUVECs were used to evaluate this property in the experiment. As HUVECs are much more sensitive to the environment as compared to L929 cells, the base solution was altered to a collagen-based solution instead. Also, the use of a collagen-based solution further helps evaluate the stability of the structure over longer periods of time (30 mins). The use of HUVECs would help to determine if the support material would affect the cell differentiation and other cellular functions. On the day 10th, HUVEC cells were alive and expressing cell markers CD31 and VWF. This indicates that the HUVECs still have the function of an Endothelial cells²⁴⁷⁻²⁴⁹. The HUVEC cells did indicate signs of fusion as their metabolic activity was lowered continuously since the second day (**Figure 5-11**). Another possible reason is due to the shrinkage of the collagen sample, thus causing the fluorescence signal to overlap. However, a 12 days study showed that the cells are uniformly distributed on the surface of the collagen scaffold with no evidence of clumping. This can be seen from the H&E staining. Signs of collagen remodelling can be observed on the day 4th as the collagen hydrogel shrunk and turned opaque²⁵⁰.

Figure 5-11: Metabolic activity of HUVECs In Collagen from day 2-6 (n=3).

Figure 5-12: SEM image of HUVECs embedded in collagen Type I at different magnifications: (A) x250 (B) x400 (C)x1000 and (D) x2000.

Figure 5-13: (a) F-actin on day 4 indicating initial phase of differentiation Scale: X20 (b) F-actin on day 12 indicating mature stage of differentiation Scale: X20 (c) Hematoxylin and Eosin (Day 12) staining to indicate size and cell distribution within the hydrogel. Scale: X20 (D) Von Willebrand Factor to determine platelet adhesion functionality. Scale: X20 (E) CD31 to determine *cell adhesion functionality of HUVECs*. Scale: X20 and (F) Collagen scaffold seeded with HUVECs after Day 12. Scale: 5 mm

Evaluation on resolution and accuracy of printed construct

The resolution and accuracy of the printed construct demonstrates the effectiveness of this process of bioprinting.²⁵¹ In both the GelMA and collagen constructs, the overall features of the hydrogels are still similar to the initially printed products even after 7 days of culture. Due to the difference in stiffness and concentration different between GelMA and collagen, shrinkage in collagen can be expected to be higher. In both materials, a resolution of approximately 100 μm can be obtained achieved but it is not representative throughout the entire structure. Depending on the depth, the density of the hydrogel and the interaction between the support material, the resolution of the struts would

vary. To mitigate this differences in accuracy, the base hydrogel could be printed on every layer to reduce the differences caused by this form in indirect printing.

5.4 Summary

3D complex structures are necessary for fabricating tissues for many different applications especially for the choroidal capillaries which are complex and difficult to manufacture due to its minuscule size. Presently, there are not many processes that could produce soft 3D complex hydrogels directly onto a platform. This new process helps to produce an accurate product with a resolution of approximately 100 μm by indirectly printing the support structure for the hydrogel of interest, providing good printability, shape integrity and able to work universally.

In the in-vitro evaluation, it has been proven that this process of bioprinting has little to no effect on the cells and the printing process has low levels of toxicity as most of the cells could still proliferate until day 14. Both the GelMA samples and the collagen samples are effective platforms for cell proliferation. The HUVECs test proved that the Collagen hydrogel after undergoing the indirect printing could still support the production of VwF and CD31, showing that they are functional. The presence of the endothelial marker denotes that the hydrogel is efficient for vascularization application. In the next chapter, a proof of concept use of a hybrid membrane and bioprinted structure will be discussed.

Chapter 6: Proof of Concept- Hybrid Three-Dimensional (3D) Bioprinting Of Retina Equivalent for Ocular Research

6.1. Introduction

The retina as discussed in chapter 2 is a complex multilayered tissue that collects and decrypts information of light energy and then transmits such information to brain to reflect images of the visual environment. Retinal pigment epithelium (RPE) is a highly specialized cell monolayer with pigmented microvilli aligning the Bruch's membrane located between the neural retina and the choroid in the eye²⁵². Mature RPE cells are mitotically quiescent under the physiological condition in the eyes; however, the RPE cells start to proliferate when the neural retina suffers from traumatic injuries²⁵² [1]. Many retinal diseases may be linked to RPE degeneration. The RPE is an essential supporting tissue for nutrient transport, production of growth factors and phagocytosis of the photoreceptor outer segments²⁵³. The photoreceptor and associated retinal circuitry is a complex system, and it is challenging to be repaired through regenerative medicine^{252 253}. Several mechanisms of retina degeneration may cause outer retina deterioration, including photoreceptors and the associated RPE cells. The degeneration leads to irreversible vision loss, and currently no effective treatments exist to permanently stop the degeneration and to restore normal retinal function from lost vision.

Research on retinal regeneration of cells, including application of mature photoreceptors, progenitor cells, retinal sheets and RPE cells, and these trials demonstrate improved vision in animal studies²⁵⁴. However, the mechanism of

the visual improvement is not clear, and the data from animal trial cannot be translated into effective clinical treatments yet. During retinal regeneration, the cell organization/alignment, integration and differentiation into retinal cell types are critical to its functionality²⁵⁵. The photoreceptors/ RPE complex functional unit is vital for normal vision; furthermore, their layered organization and correct orientation are critical for normal function and survival of the retina. The implanted cells need to complete retinal integration and differentiate into mature retina cell types²⁵⁶. The implantation of the cells into the subretinal region may cause significant cell loss, and cell behaviour after implantation may not be controllable; thus, the implanted retinal cells and tissue may form abnormal rosettes²⁵⁷⁻²⁵⁹. Cell viability and differentiation are significantly improved when the cells are transplanted with scaffolds²⁶⁰. The scaffolds can provide necessary mechanical and physical supports for cell attachment, proliferation and differentiation^{125 261}. However, conventional scaffold fabrication methods lack precision, and are incapable to prepare constructs with complex designs^{125 262}. Three dimensional (3D) bioprinting can precisely deliver cells and biomolecules to prepare micro-tissues, micro-organs and mimetic extracellular matrix, which bring researchers effective strategies for the investigation of disease progression, drug metabolism and applications of tissue or organ transplantation^{125 263-265}.

Human retina is a highly complex vascularized tissue that contains at least 60 functionally different cell types, including rod and cone photoreceptor cells, horizontal cells, bipolar cells, amacrine cells, retinal ganglion cells as well as support cells, glial cells, *etc*^{252 266}. The multiple cells need to cooperate in concert

with each other to successfully relay visual signal to brain, and only specific cells need to be replaced during certain diseases, for example the retinal ganglion cells in glaucoma, or the photoreceptor cells in retinitis pigmentosa^{252 260}. Moreover, certain areas of the retina may need replacement in some conditions, for example in the treatment of age-related macular degeneration (AMD) that arises as the result of chronic and low-grade inflammation in the central outer retina, leading to the RPE and Bruch's membrane degeneration^{253 267}_[2,14]. Moreover, there are other diseases that will also cause macular disease^{252 268}_[1,15]. All the diseases lead to the malfunction of RPE and photoreceptors. In some extreme cases, the whole eyeball may need to be removed and replaced due to retinoblastoma; thus, the 3D bioprinting technology is indeed necessary to regenerate complex retina²⁶⁶_[13]. The retina models are useful for the investigation of neurogenesis regulation and cell diversification for AMD diagnosis and early treatments. There are only few attempts and reports regarding retinal regeneration and *in vitro* retina models.

In this chapter, the 3D bioprinting technology for creation of RPE (ARPE-19) and photoreceptors (Y79) retina equivalent is reported, and the printed construct may serve as a meaningful retina model for the investigation of RPE and Y79 interactions, and retina-related disease mechanism, treatment options and tissue regenerative strategies. More over both ARPE-19 and Y79 uses similar cell media and thus would remove the influence caused by incompatible cell media. This chapter will also help to prove the concept of bioprinting on a casted membrane. The use of these two technique will enable us to understand the feasibility of the potential of printing on the membrane and interaction between

the membrane and the cells after printing. This proof of concept would also path the way towards the development of a full choroidal-retina construct through the development of the use of these methods.

6.2 Materials and Methods

Materials Preparation

Hematoxylin/Eosin (HE), chloroform, polyethylene glycol (PEG, Fluka 88276), alginate (W201502), Pluronic F-127 (P2443) and calcium chloride were purchased from Sigma-Aldrich. Polycaprolactone (PCL) powder was purchased from Perstorp (Mw 50000). ZO-1 Monoclonal Antibody, FITC (ZO1-1A12,), NucBlue® Live ReadyProbes® reagents and ActinGreen™ 488 ReadyProbes® reagents were purchased from Thermo Fisher Scientific.

Cell Culture

Human retinal pigmented epithelial cell line (ARPE-19, CRL-2302; ATCC, Rockville, MD, USA) and human retinoblastoma cell line (Y79, HTB-18™, ATCC) were cultured in DMEM:F12 (ATCC) and RPMI 1640 (ATCC) media at 37 °C with 5% CO₂, respectively, and the media was supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics.

Bioink Preparation

Alginate and pluronic were exposed and sterilized in INTELLIRAY UV Flood 400, ($\lambda = 320\text{--}390\text{ nm}$; density: 115 mW/cm²) for half an hour. 10% alginate solution was prepared by the addition of 10 g of alginate powder in 100 mL double-distilled water, and the solution was incubated in 60 °C overnight. Then, the 10% alginate solution was mixed with pluronic to form a complex bioink consisting

of 2% Alginate (w/v) and 25% Pluronic (w/v). The bioink was stored at 4 °C for future applications.

ARPE-19 Cell Bioprinting

The ARPE-19 cells upon confluence were washed with phosphate-buffered saline (PBS) three times. Then, 2 ml of Trypsin-EDTA (0.25%) was added onto the cells and incubated for 5 mins. When the cells were detached from the flask, the trypsin was neutralized by 5 mL DMEM:F12 full media. The cells were counted and centrifuged, and then they were reconstituted in cell culture media at a concentration of 1×10^6 cell/ml. The cell solution was transferred into a cartridge for microvalve-based bioprinting. The bioprinting procedure was based on the drop-by-drop pattern to achieve a final seeding density of $2,786 \pm 492$ cells/cm² on an ultrathin membrane. The ultrathin membrane was fabricated according to our published protocol¹⁰²_[16]. Then, the cells on the ultrathin membrane were further cultured for two weeks.

Y-79 Cell Bioprinting

Y-79 cells were in a suspension culture, and the cells were counted before collection. The cell pellet was resuspended in alginate/pluronic bioink to obtain a final cell density of 1×10^6 cell/mL. The Y79 cell-laden bioink was filled in a cartridge and kept at 37 °C. The cartridge was connected to a 21G needle tip, and pressure (2 bars) was applied to motivate the bioink to go through the needle to print two distinctive patterns on the ARPE-19 cell-seeded ultrathin membrane: a high average cell density at the center (HC) and a high average cell density at the periphery (HP). The bioprinted Y79 cell-laden bioink was

crosslinked in 50 mmol/L calcium chloride solution for 5 mins. Subsequently, the retina equivalents were cultured in cell culture media.

Bioprinted Retina Equivalent Characterization

The cell viabilities of bioprinted ARPE-19 cells at day 1, 7 and 14 were evaluated by prestobblue (Thermo Fisher, Grand Island, NY, USA) assay in test media (1 mL) consisted of prestobblue (10%) and FBS (5%).

Control groups were cells without bioprinting. Then, the cell viabilities were calculated according to vendor's protocol. Meanwhile the bioprinted ARPE-19 cells at day 1, 7 and 14 were fixed in 4% paraformaldehyde; then, they were stained by ActinGreen™ 488 ReadyProbes® and NucBlue® Live ReadyProbes® reagents and observed under inverted microscope (Zeiss). The bioprinted ARPE-19 cells on ultrathin membrane at day 14 were also observed under inverted microscope, and the sample was then fixed by 4% paraformaldehyde and HE stained. The bioprinted samples were stained by ZO-1 Monoclonal Antibody, FITC (ZO1-1A12)/NucBlue® Live ReadyProbes® reagents and ActinGreen™ 488 ReadyProbes®/ NucBlue® Live ReadyProbes® reagents and live/dead assay, respectively. The stained samples were observed under inverted microscope (Zeiss) and laser scanning microscope (Zeiss LSM 710). For scanning electron microscope (SEM, JEOL) observation, the samples were fixed at day 1, 7 and 14, and then were dehydrated in 30%, 50%, 75%, 95% and 100% ethanol gradually before critical-point drying, and the samples were sputter-coated (sputtering time 90 s and current 20 mA) by gold before SEM observation.

Statistical Analysis

All data was presented as means $\pm$ standard deviation ($n = 3$). Statistical analysis was implemented by paired samples t -test and multiple comparisons using single factor analysis of variance (ANOVA) and post-hoc Tukey tests using SPSS Statistics version 19.0, and $p < 0.05$ was considered statistically significant.

6.3 Results

The whole bioprinting strategies for retinal regeneration could be clearly observed in **Figure 6-1**. The bioprinting protocol was designed to precisely and efficiently simulate the biological functions of native retina. The ARPE-19 cells were precisely bioprinted on the ultra-thin membrane at discrete places to obtain homogenous cell seeding, and then the cells were allowed to grow for two weeks until the formation of ARPE-19 cell monolayer. The Y79 cell-laden alginate/pluronic bioink were bioprinted on the ARPE-19 cell monolayer to achieve two different cell-seeding densities, as can be seen in **Figure 6-1 C1 and C2**. The bioprinted ARPE-19 cells were closely monitored at day 1, 7 and 14 via prestobblue assay (**Figure 6-2**). The trend of cell viability in the bioprinted samples were similar to that of the control, their values increased gradually from day 1 to day 14, and no significant differences were observed in both groups. In **Figure 6-3**, the ARPE-19 cell morphology and proliferation were investigated, and the cells attached to the membrane within 24 hours, and then migrated and spread on the membrane. The cell number increased markedly, and finally the bioprinted ARPE-19 cells formed cell monolayer at day 14. The bioprinted ARPE- 19 cells on the ultrathin membrane at day 14 were further

analyzed by inverted microscope, and the bright field image and HE staining indicated that the bioprinted cells formed an intact cell monolayer on the ultrathin membrane (**Figure 6-4**). The bioprinted ARPE-19 cells on ultrathin membrane were investigated under confocal microscope (**Figure 6-5**). It can be observed that the strong actin filaments were within each cell, and the cells were closely packed with polygonal appearance. Moreover, no cell clusters and aggregations were observed on the ultrathin membrane at week 2. Furthermore, the bioprinted ARPE-19 cells were stained by ZO-1 antibodies (**Figure 6-6**), and it showed that robust tight junctions existed within the ARPE19 cell monolayer. The Y79 cell-laden alginate/pluronic bioink was printed on the ultrathin membrane upon the formation of ARPE-19 cell monolayer. In **Figure 6-7**, the cell-laden bioink was bioprinted into two distinctive patterns: the first one is with the higher average Y79 cell-seeding density at the center, and the other one is the higher average Y79 cell-seeding density at the periphery. The samples in both groups were maintained in culture media for seven days. The Y79 cells in alginate/pluronic bioink after bioprinting were evaluated by live/dead assay, and the live cells were stained in green while dead cells were in red color (**Figure 6-8**). Most of the Y79 cells survived at day 1, and they proliferated on day 4 and 7 with high cell viability. Subsequently, the bioprinted Y79 cell-laden constructs were observed under SEM (**Figure 6-9**): the width of each construct was around 0.21 mm, and the smoothly connected constructs maintained consistent morphology in shape and size. The Y79 cells can be observed either at the surface or inside the bioprinted construct (**Figure 6-9 D-F**), and they were in “sphere” shape.

Figure 6-1: Design of bioprinting toolpath for retina equivalent (A); bioprinting of ARPE-19 cells on ultrathin membrane (B1) to obtain homogenous cell seeding (B2) and the cells finally formed monolayer within two weeks (B3); Y79 cell-laden bioink bioprinting toolpath to achieve two distinctive cell-seeding densities: high average cell density at the periphery (HP, C1) and high average cell density at the center (HC, C2).

6.4 Discussion

Severe retina degeneration due to the malfunction of photoreceptor and RPE cells may finally lead to irreversible vision loss. Current treatment methods can only delay the disease progression, while it is almost impossible to restore the full function of the deteriorated sensory retina. More importantly, it is challenging to diagnose retina-related diseases at their early stages. For example, AMD is one of the major causes of visual loss in elderly people. 5% of population age above 65 suffers AMD, while the percentage goes up gradually to 12.5% for age 80 and above^{269 270}. The AMD according to its symptoms can be divided into “dry” and “wet” conditions. Visual loss could happen within months or several years. Majority of AMD initiates as the “dry” type (80%–90% patients), and it may further deteriorate in to “wet” type (10%–20% patients)²⁷¹. The early diagnosis of AMD faces many difficulties, and the AMD is usually asymptomatic at its early stages; occasionally some patients experience acute symptoms such as vision loss, unclear vision, scotoma, visual distortion, *etc*²⁷². The disease development of AMD usually advances slowly without significant symptoms from appearing, thus understanding the disease development is largely relative to the wellbeing of the elderly people in the world.

Figure 6-2: Cell viability assay of manually seeded and bioprinted ARPE-19 cells, $p > 0.05$

Figure 6-3: Fluorescent images (F-actin) of bioprinted ARPE-19 cells at day 1 (A), day 7 (B) and day 14 (C); scale bar: 200 μm

Figure 6-4: Phase-contrast image of bioprinted ARPE-19 cells on ultrathin membrane (A), and HE staining of bioprinted ARPE-19 cells (B) at week 2; scale bar: 500 μm

Significant progress in 3D printing technology has demonstrated a potential for organizing advanced cellular and tissue structure with high physiological

relevance²⁷³. This technology is successfully applied in many biomedical applications including skin, heart and retina bioprinting, *etc*²⁷³. The 3D bioprinting technology can efficiently and precisely produce the retina equivalent that a biomimetic platform can be built (**Figure 6-1**)²⁷⁴⁻²⁷⁶. This proposed platform will be extremely useful for eye related diseases' risk assessments and drug testing. In this chapter, the ultrathin membrane is utilized to represent Brunch's membrane, which is a very thin tissue barrier (2–4 μm) between the retina and choroid. It serves two major roles: as a substratum for metabolically active RPE cell attachment and as a vessel wall. The membrane is involved in AMD and other chorioretinal diseases. The PCL ultrathin membrane enhances cell morphology and barrier formation of ARPE-19 cells, and the stronger and more uniform tight junctions can be observed among ARPE-19 cells on the PCL membrane than that of a transwell membrane¹⁰². The ultrathin membrane is successfully utilized in this research to support ARPE-19 cell seeding, proliferation and formation of monolayer. The bioprinting process does not compromise ARPE-19 cell viability (**Figure 6-2**).

The bioprinted ARPE-19 cells show interesting migration patterns on the ultrathin membrane (**Figure 6-3**); the cells remain inside the bioprinting droplets at the first 24 hours and subsequently the cells migrate, proliferate and gradually occupy the gap among each droplet, until finally an intact ARPE-19 cell monolayer is formed on the ultrathin membrane. The high quality of ARPE-19 cell monolayer is verified by confocal microscopy and by HE and ZO-1 staining. The cells cover the whole membrane, and no vacant area is observed (**Figure 6-4**). In the confocal image, the actin staining indicates intense interactions among

cells, while DAPI staining (cell nucleus in blue) proves that no overlaid cells are in the cell layer (**Figure 6-5**). Thus, a high quality ARPE-19 cell monolayer is created on the ultrathin membrane. The vital function of the RPE is to control the ionic composition of the subretinal region, subsequently providing sensory retina the biological environment for its proper function. The sensory retina-related diseases are very subtle and hard to be discovered at their earlier stages; although many scientists prefer to use fresh samples as their experimental model, the available samples are quite limited, especially when they are collected from human²⁷⁷. On the other hand, animal models may provide alternatives; however, the animal models are not fully controllable and the experimental data is not fully translatable²⁷⁸. Therefore, cell culture and tissue engineering offer significant flexibility to create *in vitro* retina tissue models and to study the mechanism of retinal regeneration and disease development. The 3D bioprinting technology offers powerful tools for tissue model creation to fully mimic human retina. In this article, the ARPE-19 cell-seeded ultrathin membrane represents Bruch's membrane and RPE monolayer with tight junctions (**Figure 6-6**), subsequently the Y79 cell-laden bioink is printed on the ARPE-19 cell monolayer to achieve two distinctive patterns (**Figure 6-7**). Pure alginate bioink has shown excellent cytocompatibility²⁷⁹—however the bioink has poor printability. Pluronic is thermoreversible and generally nontoxic, and it has been employed for drug delivery including intramuscular, intraperitoneal and subcutaneous injections²⁸⁰. Therefore, the alginate/pluronic complex bioink is prepared to maintain excellent biocompatibility and achieve improved printability. Human photoreceptors are composed of cone and rod cells, and the

density distribution of the cone and rod cells are regulated from the foveolar to retinal periphery, with the highest cone concentration is observed at the foveola while rod density is at its maximum density at a 5–6 mm from the foveola. Y79 cells express both cone- and rod-specific antigens²⁸¹⁻²⁸³, and fresh retinoblastoma tumor cells can differentiate to photoreceptor, neuronal and glial cell lines^{284 285}. Therefore, Y79 cells are useful for retina-related research²⁸⁶ in 3D cell-bioprinting to investigate biological responses of sensory retina cells during and after bioprinting. The bioprinted retina constructs are cultured in media for seven days, and the bioprinted Y79 cell-laden bioink can preserve its basic configuration during the culture period. Although the complex bioink is crosslinked by calcium ions that are slowly released in culture media, its structure may gradually deteriorate. Therefore, the culture media should be carefully and gently removed to avoid structural disruption. The Y79 cell viability is not compromised in the bioink, and the cell density increases from day 1 to day 7 (**Figure 6-8**). In SEM images (**Figure 6-9**), the Y79 cells can be easily observed on the surface of the bioprinted cell-laden hydrogel; the inner structure of the cell-laden bioink is porous and allows transportation of nutrients and waste, thus providing a benign environment for Y79 cell reproduction while maintaining biological interactions with the underneath ARPE-19 cell monolayer.

Figure 6-5: Confocal images of the bioprinted ARPE-19 cell monolayer on ultrathin membrane; F-actin in green and cell nucleus in blue, with the x-y projections of single optical section is presented in the central image with respective side-views on x-y and y-z (bottom and right) axes; scale bar: 100 μm

Figure 6-6: ZO-1 and DAPI staining of bioprinted ARPE-19 cell monolayer on ultrathin membrane at week 2; scale bar: 20 μm

Figure 6-7: The bioprinted retinal equivalents with two distinctive Y79 cell-seeding density: high average cell density at the center (HC, A) and high average cell density at the periphery (HP, B); *: central area, **: periphery; scale bar: 10 mm

Figure 6-8: Live/dead assay of Y79 cell in bioprinted alginate/pluronic complex bioink at day 1 (A), day 4 (B) and day 7 (C); scale bar: 200 μ m

Figure 6-9: SEM images of bioprinted Y79 cell-laden alginate/pluronic complex bioink (A, B and C); Y79 cell distribution at the surface of bioink (D), and the Y79 cell distribution

at the cross sections of the alginate/pluronic complex bioink at magnification of 200× and 1000× (E and F); yellow arrows indicate Y79 cells

6.5 Summary

A retina equivalent was prepared by hybrid 3D bioprinting and reported in this chapter, and the bioprinted construct was composed of ARPE-19 cell monolayer on ultrathin membrane, and a third layer with two distinctive patterns (Y79 cells in alginate/pluronic bioink). The cell viability, cell morphology and quality of the bioprinted ARPE-19 cells were closely investigated, and the ARPE- 19 cells formed high quality monolayer on the ultrathin membrane within 14 days. The Y79 cell-laden alginate/ pluronic bioink was successfully bioprinted on the ARPE-19 cell monolayer with two distinctive patterns. The Y79 cells in the bioink showed benign morphology and proliferated in seven days. The bioprinted retina equivalent has acceptable cytocompatibility with advanced structure aiming to simulate native retina. Therefore, it may have broad applications in retina-related research including investigations on disease mechanism, drug testing and treatment methods.

Chapter 7 Conclusion and Future Work

7.1 Conclusions

The research has fulfilled the main objective of this project: Development of an ultra-thin biocompatible polymer membrane to simulate Bruch's membrane for adhesion of RPE cells; Development of novel process for bioprinting of supported hydrogel bioprinting for vascularization; Prove of concept using hybrid bioprinting and membrane fabrication to develop a retina complex. The main contributions of this research are as follows:

- 1) A synthetic Bruch's membrane was fabricated using a drop casting technique. This scaffold enhanced the ARPE-19 cell morphology and the barrier formation compared to the cells on the transwell membrane or on regular well plates. The ARPE-19 cells on the PCL membrane exhibited more uniform tight junctions indicating a well-developed barrier. The formation of this barrier is essential in many homeostatic functions including fluid transport and formation of a blood-retinal barrier. The development of a PCL membrane in this work could provide critical success for the future RPE transplantation.
- 2) A bioprinting strategy based on the bioprinting of supported hydrogel bioprinting for vascularization was devised to improved the print resolution without impacting on cell viability. This new process helps to produce an accurate product with a resolution of approximately 100 μm by indirectly printing the support structure for the hydrogel of interest, providing good

printability, shape integrity and able to work universally. Also, process of bioprinting has little to no effect on the cells. Both the GelMA samples and the collagen samples are effective platforms for cell proliferation. The HUVECs test proved that the Collagen hydrogel after undergoing the indirect printing could still support the production of VwF and CD31, showing that they are functional. The presence of the endothelial marker denotes that the hydrogel is efficient for vascularization application.

3) A proof of concept of using a hybrid bioprinting technique that combines the use of bioprinting and drop casted membrane in the fabrication of a retina complex was identified and developed. The printing of cells on the membrane was reported and formed high quality monolayer on the ultra-thin membrane. The use of Y79-seeded hydrogel on the RPE monolayer was able to produce an acceptable cytocompatibility and simulate the native retina.

7.1.1. Fabrication of Ultrathin, Free-Standing and Porous Polymer Membranes for Retinal Tissue Engineering

A fast and easy method to create a free-standing, porous and ultrathin PCL membrane, through drop casting the polymer blend on the liquid interface was fabricated. We found that the phase separation phenomenon occurred during the fabrication process resulted in a porous membrane like the Bruch's membrane. The synthesised PCL membrane was found to have regular and interconnected pores, indicating that adequate nutrient exchange can take place across the membrane even when the media is separated from the membrane. This was a unique feature of the synthesised membrane as against

the previously reported phase separated membranes. Cell viability of the membrane was established and found to be like that of a PET membrane. The membrane was also reported to have low cytotoxicity. The results from this study suggested that the porous PCL membrane boosted the ARPE-19 phenotype compared to cells grown on transwell inserts and plate. It was possible that the ultrathin characteristics together with the pits and pores of the membrane could have improved ARPE-19's cellular behaviour, thus enhancing the superiority of the membrane as a possible substrate for in-vitro culture. Work on fhRPE cells has also provided similar results in terms of the enhanced functionality of the RPE cells when the scaffold was porous¹⁹³. The functionality of the RPE cells in terms of the ability to phagocytose photoreceptor outer segments (POS) and protein secretion have not been studied on this platform. In the future, combined with collagen grafting, this work would enhance and enable the fabrication of a complete RPE tissue model or an implantable engineered tissue. Furthermore, the use of new processes such as bioprinting¹⁹⁴⁻¹⁹⁶ and 3D printing could help to control the reliability and high throughput production of these membranes and tissue constructs.

This study demonstrated the fabrication of an ultrathin PCL membrane that could mimic the Bruch's membrane. This scaffold enhanced the ARPE-19 cell morphology and the barrier formation compared to the cells on the transwell membrane or on regular well plates. The ARPE-19 cells on the PCL membrane exhibited more uniform tight junctions indicating a well-developed barrier. The formation of this barrier is essential in many homeostatic functions including fluid transport and formation of a blood-retinal barrier. The development of a

PCL membrane in this work could provide critical success for the future RPE transplantation.

7.1.2 Development of a versatile, universal indirect bioprinting process for high resolution bioprinting

In this research, an indirect method of bioprinting has been successfully developed. By using an alginate-pluronic support structure, low viscosity cells-laden hydrogels can be fabricated. The interaction of Ca^{2+} and alginate helps to prevent osmosis interaction between the two hydrogels (model and support structure). The long-term stability of the support material helps to enable sufficient mechanical strength for any form of low viscosity hydrogel including GelMA and Collagen Type I hydrogels. This helps to open the bioprinting system to more fundamental hydrogels used in the research community without the need to fabricate new types of hydrogel. Although the support material is stable, the support structure can be easily remove through the use of cold PBS to without harming the cells.

From the in vitro evaluation, both L929 and HUVECs were survived over 7 days in all the samples. The cells were able to migrate, proliferate and showed the sign of functionality. Both cells are able to attach and pack together. The presence of actin showed that the cells are fusing while the presence of CD31 and VWF showed the endothelium cells' functionality as well as efficiency of vascularization. This provided that the indirect bioprinting and support material support cell growth and cell differentiation.

This process of printing helps to enable the printing of more desirable hydrogel without the need to be concern about the printability of the cell-laden hydrogels. This is especially important as currently; the number of printable hydrogels are limited. The use of this process would possibly enable the native ECM of the cell to be printable. The process produces hydrogel structures with good printability with resolution of up to 100 μm . Also as the support materials (pluronic and alginate) have been heavily researched to be used in tissue engineering, the safety concern of these temporary supports would possibly have little to no long-term effects on the tissue. Since the base hydrogel was casted after printing of the support material, the base hydrogel would generally have no issues with delamination and thus would tend to have more isotropic tensile strength. Therefore, this work offers a great potential for future bioprinting hydrogels and do not limit the type of hydrogels for tissue engineering and biomedical applications. With the advent of the use of microvalve technology, the resolution of the printed structure could be further enhanced to cater for smaller features if required.

Scaling up of bioprinted products by increasing the size of the print would generally face multiple issues, deformation of the parts are more significant in larger scaffolds due to the additional weight acting on the hydrogel, additional research is required to improve the rheology and the mechanical strength of the hydrogel²⁸⁷. The current process of printing enables the printing of up to 1 cm thick with little distortions (10-50 μm). Further improvements such as a system to vary the pressure, print speed to compensate for the evaporation could enable scaling up of the process to accommodate a larger printed structure.

Compared to traditional methods, the process requires less human intervention and the results has higher repeatability.

7.1.3 Proof of Concept- Hybrid Three-Dimensional (3D) Bioprinting Of Retina Equivalent for Ocular Research

Till date, there is no full retina tissue that has been fabricated. Most of the research thus far has been focused on the fabrication of the artificial Bruch's membrane but not the choroidal capillaries for a complete tissue model. The role of the choroidal capillaries enables the study of neovascularisation of the retina which is especially significant in studying of the retina condition. This study has shown the feasibility of the use of a hybrid fabrication technique that encompass the use of a drop casted membrane and bioprinting. Bioprinting of hydrogels on the membrane were demonstrated. It also demonstrated the capability of bioprinting's ability to accurately place cells in a specific location. There is potential to further improve this technique to encompass the use of endothelial cells to build a complete retina complex.

This study also provided some insight in the interactions between Y79 cells and ARPE-19 cells. Further improvements to this technique can be done using native hydrogel such collagen as it would enable better cell proliferation and improve the cell-cell interactions.

7.2 Future Research Directions

7.2.1 Usage of native ECM as base for indirect bioprinting

In native tissues, the ECM structures are not only made of collagen but also other components such as proteoglycans, polysaccharides and elastin. The

changes in these components affects the tissue physiological properties. Thus, by systematically optimising the components we would be able to optimally regulate the important cell processes including migration, differentiation, proliferation and apoptosis. One example is through adjusting the collagen and elastin concentration of a hydrogel. These changes would affect the ECM's stiffness and elasticity. These changes in stiffness affects the traction force on the cells during adhesion as they pull on the ECM. This action of pulling and reassembly of focal adhesion leads to signal transduction and cellular response which remodels the cell's behaviour.

The use of pure collagen would not be a good representation of an ECM matrix as the remodelling of the collagen by the cells changes the structure of the collagen over time. These changes do not provide a stable structure for the cells which would affect the functions of the cells. The use of the above indirect method of printing not only allows multiple components on the ECM to be included in the printing but also with minimal change in their composition with the addition of calcium ions into their composition as compared to other forms of indirect printing. By combining the use of this indirect method of the use of ECM, we would be able to produce a better tissue sample that could provide a better representative tissue model.

7.2.2 Dynamic co-culturing on hydrogel structure

The hydrogel structure discussed in chapter 6 showed that the HUVEC cells migration towards the surface before fusion. To improve the efficiency of vascularisation, the hydrogel can contain more than one type of cells. One

example is the use of human fibroblast cells and HUVECs cells for the formation of a blood vessel. Both the fibroblasts and HUVECs can be mixed into the collagen before casting onto the printed structure. Also, the use of fibroblast can also help to direct formation of blood vessels at specific points of the structures to induce the blood vessel formation. The use of a co-culturing technique will help to improve the speed of vascularisation and could result in blood vessels being embedded in the hydrogel as opposed to the current HUVECs forming the blood vessels on the surface of the hydrogel.

Another method to improve the efficiency of tissue formation is the use of dynamic culturing. The use of a perfusion reactor will help to increase the nutrient uptake of the cells by increasing the nutrient concentration on the surface of the hydrogel. This will aid in increasing the proliferation rate and avoid cell death due to hypoxia. However, the use of a dynamic culturing system requires knowledge in perfusion system as changes in the flow rate would affect the cells behaviour and survival. Another factor to consider is that as the surface of the hydrogel is mostly hydrophilic, the flow through the hydrogel pores would require higher dynamic pressure as compared to a hydrophobic surface.

Once optimised the use of both a co-culture and a dynamic cell culturing system. The efficiency of producing blood vessels could be vastly improved. The use of such a technique would help to reduce the time required for the cells to form a tissue and would help to reduce the time to create drug models.

7.2.3 Fabrication of a full retina-choroidal tissue model

As discussed earlier, the retina is made of a multitude of cell types, ranging from RPE cells to photoreceptor cells (rod and cone cells). To produce a complete tissue model, the model would require neuronal cells such as the rods and cones to shed their outer segments. The detection, production and degradation of these outer segment would provide a better understanding of the retinal tissue. Current method of such detection can only be done via an adaptive optics optical coherence tomography (AO-OCT) on patients. Thus, these diagnostic tools can only provide an insight on the retina only when it has affected the patients. The lack of an in-vitro human testing sample has led to drug and healthcare companies being incapable to provide a conclusive result on the drug potency on the patients. However, there are challenges in producing and procuring photoreceptors cells as the cells are difficult to harvest as the retina contains multiple cells and the cells are extremely delicate. If these challenges in obtaining these cells are realised, the formation of a complete tissue model could provide a better understanding to ophthalmology and provide a much better grasp of the retina tissue. In order to fully investigate retina diseases such as Wet AMD, the combination of the use of RPE cells, HUVECs and photoreceptor in a printed structure is necessary.

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