Laser-based 3D printing using bessel beams for tissue engineering applications

Liew, Andy Wen Loong

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**LASER-BASED 3D PRINTING USING
BESSEL BEAMS FOR TISSUE ENGINEERING
APPLICATIONS**

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SCHOOL OF MECHANICAL AND AEROSPACE ENGINEERING

2020

LASER-BASED 3D PRINTING USING BESSEL BEAMS FOR TISSUE ENGINEERING APPLICATIONS

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School of Mechanical and Aerospace Engineering

A thesis submitted to the Nanyang Technological University in
partial fulfillment of the requirement for the degree of Doctor of
Philosophy

2020

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.

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Prof Huang Weimin

Authorship Attribution Statement

This thesis contains material from four papers published in the following peer-reviewed journals or accepted at conferences in which I am listed as an author.

Chapter 2 is published as: **Liew, Andy Wen Loong, and Yilei Zhang “In vitro pre-vascularization strategies for tissue engineered constructs – Bioprinting and others.” International Journal of Bioprinting (2017) 3 3-17.**

The contributions of the co-authors are as follows:

- Yilei Zhang provided the project direction, supervised the project from start to completion, provided scientific advice, and reviewed and edited the manuscript drafts.
- I prepared the manuscript drafts, conducted the literature review, and collated the information.

Chapter 3 is published as: **Liew, Wen Loong Andy, and Yilei Zhang. “Laser-based fabrication of 3D hydrogel constructs using bessel beams.” Bioprinting (2018) 9 44-51.**

The contributions of the co-authors are as follows:

- Yilei Zhang generated the project idea, provided scientific advice and guidance, supervised the project from start to completion, provided access to all equipment and materials used, and reviewed and edited the manuscript drafts.
- I performed all experimental work (including material synthesis and equipment set-up), conducted the literature review, and prepared the manuscript drafts.

Chapter 5 is published as:

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(Conference paper) **Liew, Andy Wen Loong, and Yilei Zhang. "Fabrication of lumenized vascular tubes for tissue engineering applications." Proceedings of the 2nd International Conference on Progress in Additive Manufacturing (2016).**

The contributions of the co-authors are as follows:

- Yilei Zhang suggested the project scope, provided scientific advice and instruction, provided access to imaging equipment (microscopes) and materials, supervised the project from start to completion, and reviewed and edited the manuscript drafts.
- I performed all experimental work (including material synthesis, equipment usage, cell culture, imaging, cell-staining assays), conducted the literature review, and prepared the manuscript drafts.

02 Jan 2020

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Liew Wen Loong Andy

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Summary

The ultimate goal of tissue engineering and regenerative medicine is the successful fabrication of functional tissue in the lab for implantation into patients to treat damaged tissue and organs. Over the years, 3D printing technology has emerged as a promising technique to achieve this goal as it offers superior control over construct architecture and cell placement. Scientists today are continually seeking to improve the technology in order to produce more complex biofunctional tissue constructs which eludes the tissue engineering community to this day. Scientists are also faced with the challenge of implant pre-vascularization which is deemed essential for long-term efficacy of the tissue construct post-implantation. This has led to the development of a variety of vascularization techniques in recent years.

In this dissertation, a laser-based 3D printing technique using Bessel Beams was studied for applications in tissue engineering. Compared to conventional fabrication techniques, the Bessel Beam (BB) printing technique demonstrated unique advantages which includes easily customizable construct architecture, reduced fabrication time, high print resolution, and the ability to print high aspect ratio tubular structures with anatomical relevance without the need for support material.

Through the characterization of endothelial cells encapsulated within hydrogel matrix, It was found that while collagen gel was capable of supporting cell viability and inducing endothelial sprouting, these self-assembled endothelial networks were only found on the 2D substrate surface and not within the 3D gel matrix. These networks were also found to be non-lumenized, which contradicts some reports found in the literature. It was also found that encapsulation of endothelial cells within spatially confined collagen gels is necessary for self-assembled endothelial cord formation. These results laid the foundation for subsequent cell printing experiments.

Live endothelial cells were then successfully encapsulated within hydrogel fibers using the BB printing technique and shown to possess superior cytocompatibility compared to conventional extrusion and inkjet-based printing systems. High resolution fibers down to 25 μm were printable using the BB technique, a significant improvement compared to conventional microfluidics and wet spinning techniques, and the capability to tune the fabricated fiber diameter based on mathematical models was also demonstrated. Finally, endothelial cells encapsulated within cell-laden hydrogel fibers were shown to self-assemble into endothelial cords in vitro which is desirable for pre-vascularization applications.

Overall, the work presented in this dissertation solidifies the BB printing technique as a promising fabrication approach with significant applications in tissue engineering, particularly in the field of the vascularization of tissue constructs.

1. Introduction

1.1 Background

There is a wide spectrum of human pathologies which plague mankind and affect our quality of life. These diseases often lead to organ failure and death of the patient. The conventional treatment for organ failure would be an organ transplant, where the patient's damaged organ is replaced by a functional, compatible donor organ. Although organ transplantation has proven its efficacy over the years, thousands of people still continue to lose their lives every year due to organ failure. The reason for this is the demand and supply disparity of donor organs. The demand for donor organs far exceeds the supply, and there are simply not enough donor organs to go around. According to research by the U.S. department of health and human services[1], the percentage gap between the number of patients on the waitlist and the number of organ donors has been steadily increasing every year – the number of patients on the waitlist continues to grow while the number of transplants performed and donors recovered remain relatively constant. In the U.S. alone, as of March 2018, there are more than 100,000 patients on the waitlist with another patient added to the waitlist every ten minutes. This issue is heightened in poorer, less developed countries where people simply cannot afford the expensive treatment, giving rise to black market organ trading. In recent years, there have even been reports of organ stealing where functional organs are taken from unwilling victims to be sold in the black market. These are serious problems which need to be addressed. Tissue Engineering (TE) has shown promising signs to be a potential solution to this problem.

TE is a field of research which aims to restore or replace damaged tissue and organs in patients. A common technique used clinically today to treat small areas of damaged tissue is to use autologous tissue grafts. For example, burn victims are often treated

by grafting skin from another site on their body and reapplying the graft on burn sites. However, this method is ineffective for treating large wounds as supply of autologous tissue grafts are limited. Using the TE approach, autologous tissue is grown in a lab, rather than grafted from a donor, and implanted into the patient's defect site upon tissue maturity to treat the injured site. Research in this field has led to successful engineering of simple functional tissues such as skin, cartilage, and bone which have successfully transitioned into clinical and commercial use[2]. The aforementioned tissues which have successfully made the transition are avascular which makes them easier to engineer compared to vascular tissues such as heart and kidney tissue which are more complex[3].

Most of the tissues in our body are vascular in nature, meaning that an interconnected network of perfusable blood vessels runs through the entire tissue. This vascular network is responsible for the supply of necessary nutrients through blood perfusion which keep the surrounding cells viable and functional, and also for the removal of metabolic waste produced by the cells. To engineer vascular tissue, the inclusion of a functional vascular network is vital to prevent cell necrosis and failure of the tissue. Without a vascular network, cells deep within the tissue construct will not have access to sufficient nutrients and metabolic waste removal and will eventually die; hypoxia (insufficient oxygen) is a common cause of cell death [4]. It has been found that cells located more than 150-200 μ m away from the nearest blood vessel receive insufficient nutrients for survival due to limits of diffusion[5]. This has limited the success of TE to thin sheets (2D) of avascular tissue, which rely solely on the infiltration of nutrients from its surroundings through diffusion to support cell survival. Thus, in order to successfully engineer more complex tissues/organs (which more accurately represents the majority of native human tissue), a functional, perfusable vascular network must be incorporated into the tissue constructs. For this

reason, many research groups have attempted to engineer functional blood vessel networks within their tissue constructs (known as pre-vascularization) using a variety of different techniques, and the physical and chemical cues promoting vascularization are being studied[6]. The development of a technique capable of producing in vitro pre-vascularized tissue constructs would undoubtedly be of significant value to the advancement of TE.

Additive manufacturing (AM) or 3D printing is a bottom-up fabrication approach where 3D constructs are effectively 'printed' layer-by-layer using a variety of techniques to eventually form a customizable construct based on Computer-aided design (CAD) drawings. The development of bioprinting stems from preceding AM techniques, and was developed for the printing of living tissue i.e. live cells for TE applications[7]. Today, many different bioprinting techniques have been developed and results show that the technique does indeed offer a promising way to fabricate customizable tissue constructs consisting of living cells to replace damaged tissue in patients, which could possibly replace the current gold standard of donor organ transplant[8]. Bioprinting offers several advantages compared to conventional methods to produce tissue constructs, including patient-specific customization and multi-cell type printing, and has been cited as potentially being the prominent biofabrication technique used for future research in the field of TE, although there are still multiple challenges which need to be addressed for this to be realized [9].

1.2 Objectives

The primary objective of this dissertation is to fill in research gaps in the literature on the utilization of a 3D printing technique using Bessel Beams. The research gaps are as follows:

- There are no reports with detailed studies on the advantages of the BB printing technique compared to existing additive manufacturing techniques.
- There are no reports in the literature that study the use of BBs for applications in the field of TE.
- The use of BBs to fabricate hydrogel constructs, which are a commonly used material in TE, has not been reported in the literature.
- Moreover, the use of BBs for the direct printing of live cells has also not been reported.

The objectives of this dissertation can be further subdivided into three parts:

1. To advance the field of 3D printing by investigating the advantages of a technique using Bessel Beams over conventional techniques.

3D printing techniques today require long processing times, and have geometrical limitations (i.e. aspect ratio) on printable products. Bessel beams (BBs) have been studied for various applications, mostly in material processing, with numerous reports found in current literature. The goal is to investigate the unique characteristics of BBs for 3D printing to address the aforementioned shortcomings of current fabrication techniques.

2. To determine the factors that affect endothelial network formation in cell-laden hydrogel.

In order to apply the BB printing technique for the direct encapsulation of live Endothelial Cells (ECs), it is first required to determine how the cells behave in the hydrogel material. This will provide clarity on the biocompatibility of the material, and how well the material is able to support self-assembled endothelial network formation. For the advancement of TE, it is also necessary to further our understanding of how ECs form networks in bulk hydrogel and the factors that affect the process. A better understanding of these factors will enable us to determine the optimal conditions required for endothelial network formation in

vitro, and thus bring us a step closer to producing 3D vascularized tissue constructs in the future.

3. To employ the BB printing technique for the direct cell encapsulation of live ECs in order to induce in vitro endothelial cord formation.

By combining the unique advantages of the BB printing technique, and the knowledge of how ECs behave in hydrogels material, the aim is to directly print cell-laden hydrogel constructs for the formation of in vitro endothelial cords which has significance in the field of in vitro pre-vascularization of TE constructs.

1.3 Scope

This dissertation investigates the capabilities of a laser-based 3D printing technique utilizing BBs. The printing technique is used for the fabrication of customizable 3D hydrogel constructs with applications in TE. The technique is studied to ascertain its advantages and disadvantages compared to existing fabrication techniques. All experimental procedures, equipment, and materials used in the printing technique are detailed for reproducibility.

The effects of hydrogel Extra-Cellular Matrix (ECM) material composition, concentration, and geometry on cell viability, network morphology, and lumenization are studied by encapsulating Human Umbilical Vein Endothelial Cells (HUVECs) in collagen hydrogel and subsequent characterization after culturing. Encapsulated HUVECs are characterized through various staining and microscopy techniques. Significant findings and observations are presented.

Based on the results gathered from the cell encapsulation studies, the 3D printing technique is then used for the direct cell encapsulation of HUVECs in collagen-derived,

photopolymerizable Gelatin Methacryloyl (GelMA) hydrogel fibers. Encapsulated HUVECs are cultured and characterized at specific time points to determine viability, proliferation, and morphology. The ability of the encapsulated HUVECs to self-assemble into endothelial cords in vitro is studied. The advantages as well as limitations of using this technique for TE applications is discussed.

1.4 Outline

This dissertation is organized into three main sections: (1) Literature Review, (2) Experimental Work, (3) Conclusions and Future Work. The work presented is broken down into separate chapters and provided to the reader in a step-by-step manner where each chapter leads to the next in a smooth flow of information, as illustrated in Figure 1.1. The main sections are divided into chapters as follows:

Literature Review

(Chapter 2) A critical review of the current literature will be presented. This is to provide the reader a clear overview of the research progress in the relevant areas pertaining to this dissertation including TE, In vitro vascularization techniques, Bessel Beams, and Endothelial cords. Research gaps that need to be addressed will be identified from the literature and discussed.

Experimental work

(Chapter 3) This chapter describes in detail the laser-based Bessel Beam (BB) printing technique utilized in this dissertation including all equipment, methods, and materials used for printing. Studies on the use of the BB printing technique in the field of tissue engineering has not been reported in the literature. Acellular hydrogel was used as the print material for studying the capabilities of the printing technique, without the inclusion of live cells, and sets the stage for subsequent chapters where the technique

is used for direct cell printing (Chapter 5). Acellular 3D hydrogel constructs (tubular constructs and scaffolds) were fabricated using the printing technique which have applications in TE. Advantages as well as limitations of the technique, including shortened fabrication time and customizability among others, are discussed.

(Chapter 4) The focus of this chapter is shifted away from how the printing technique works (Chapter 3), and goes into the study of how HUVECs behave when encapsulated in hydrogel ECM. As the next chapter (Chapter 5) aims to use the BB printing technique for direct cell printing, this study is necessary to understand how ECs would behave after encapsulation in hydrogel material. This chapter studies how ECs are affected by their surrounding hydrogel ECM environment, and the factors that affect cell viability, self-assembled endothelial network formation, and endothelial lumenization. This study is used to determine the print parameters (i.e. hydrogel material and construct design) to induce the formation of in vitro endothelial cords in preparation for the next chapter.

(Chapter 5) Unlike Chapter 3 where acellular hydrogel constructs were fabricated, in this chapter, live cells (HUVECs) are incorporated into the printing procedure. The aim is to use the results from both Chapter 3 and Chapter 4 to successfully fabricate in vitro endothelial cords, which addresses the need for pre-vascularization strategies in TE. Live HUVECs are encapsulated within hydrogel material using the BB technique in Chapter 3, cultured for several days, and characterized based on viability, proliferation, and morphology. The ability of the cells to self-assemble into endothelial cords is demonstrated through cell staining and confocal imaging. The use of BBs for the encapsulation of live cells has not been previously studied in the literature.

Conclusions and Future Work

(Chapter 6) This chapter provides a summary of all work done in the experimental works chapters (Chapter 3 to 5), results, and significance. The conclusions from the experimental work chapters are combined and presented in terms of contribution to the field of TE, as well as the prospective direction of future research in this area.

Figure 1.1. Dissertation outline; breakdown of individual chapters and work flow. Literature review (green) is presented in Chapter 2, Experimental work (blue) in Chapters 3 to 5, and Conclusions and future work (orange) in Chapter 6.

2. Literature review

Work presented in this chapter is largely based on the following publication: **Liew, Andy Wen Loong, and Yilei Zhang “In vitro pre-vascularization strategies for tissue engineered constructs – Bioprinting and others.” *International Journal of Bioprinting* (2017) 3 3-17.**

2.1 Tissue Engineering

2.1.1 Overview

The term *tissue engineering* (TE) was first coined in 1993 by Langer and Vacanti in their highly influential paper[10]. TE is a cross-disciplinary field of research, comprising the principles of biology and engineering to create functional tissue in a lab. These fabricated tissues can be applied in the field of regenerative medicine for the replacement, repair, and restoration of damaged tissue in a patient. A schematic diagram is presented in Figure 2.1 to illustrate the general principles of TE. A patient suffering from tissue injury, such as skin burn, can be treated using this approach. A biopsy is first performed to extract healthy autologous cells (stem cells or skin cells) from the patient. These cells are treated and expanded in vitro to obtain a sufficient number of functionally differentiated cells needed for the treatment. The cells are then seeded onto a suitable biodegradable scaffold, which acts as a support structure for cell adhesion and provides a conducive environment for cell growth. When the tissue-engineered graft is fully matured, it can then be transplanted onto the patient's injury site to enhance the wound healing process. The use of the patient's autologous cells reduces the immunological response and rejection of the graft by the host, which is a persistent problem for the TE community[11].

Figure 2.1. Principles of tissue engineering. (Adopted from ref.[12])

Research in this field is expanding quickly and has given rise to many TE companies which carry out research and manufacture human Tissue-Engineered Products (hTEPs) for clinical use today. In Singapore, due to the possible negative health effects, these products are regulated by Health Sciences Authority under the Medicines Act to ensure the safety of consumers[13]. TE is still in its early phases, thus only few hTEPs have been successfully translated into commercial availability. These commercially available hTEPs mainly comprise skin, cartilage, and bone products. By 2003, more than 20 skin replacement products were available in USA and Europe[2]. Products such as Cultured Epidermal Autografts (CEA, Epicel) used to treat severely burned patients showed effectiveness and positive results from 5-year clinical trials[14]. Tissue-engineered cartilage products have also found application today to treat traumatic knee joint damage by undergoing Autologous Chondrocyte Transplantation (ACT) surgery[2]. Clinical results demonstrated significant improvement in patients implanted with Carticel and HyalograftC after 6 months with newly formed tissue

possessing similar structural features with native cartilage tissue[15]. The application of tissue-engineered bone products is limited to the treatment of small bone lesions as larger defect sites still remain untreatable by this approach and autologous bone grafts still remain the preferred approach. With the successful translation of these hTEPs into clinical application, TE has proven its legitimacy as a promising candidate for the treatment of injured tissue, sparking more research in the field. Today, research groups all over the world are working to create hTEPs from various tissues such as cardiac[16–18], liver[19], cornea[20], trachea[21,22], esophageal[23], and arterial[24] tissue among others.

Many groups have published works on the successful engineering of functional tissue in vitro, such as bladder tissue[25], although they have not yet been used in clinical trials due to its early stage of development. Many of these published works report the successful engineering of only thin, microscale tissue constructs. The reason for this, as previously mentioned, is due to the fact that cells deep within a large tissue construct have limited access to nutrients and metabolic waste removal, causing them to die during long term culture. With the ultimate goal of TE in mind, which is to successfully engineer complete organs, techniques need to be developed to allow the fabrication of larger tissue constructs. The vascularization of thick TE constructs is deemed to be vital to the progress of TE, and remains a great challenge today[26–29].

2.1.2 Vascularization

With avascular tissue products such as skin and cartilage already made commercially available, tissue engineers are now looking to engineer larger, more complex tissue which could potentially be used as a viable treatment option for patients suffering from critical diseases[30]. However, there are a number of crucial challenges to be addressed to meet this goal[31]. Our native tissues possess highly specific

architectures, and different tissues have their own unique structural organization[32]. It is imperative that tissue engineers accurately emulate the intrinsic heterogeneous architecture of these complex tissues when fabricating TE construct as there is a well-accepted correlation between tissue architecture and pathogenesis[33]. The successful engineering of complex tissue also requires control of differentiated function of the cells within the TE construct, failing which could cause the TE construct to be dysfunctional as cells do not carry out the specific functions that they should, thus rendering the construct useless and malignant. There are many other challenges in the pursuit of engineering large, complex tissue constructs i.e. matrix stiffness, molecular gradients, hierarchical structure[34]. One of the critical challenges identified by experts in the field is addressed in this dissertation; the vascularization of TE constructs.

As previously mentioned, the incorporation of a mature, inter-connected vascular network within a tissue construct prior to implantation is vital in TE as it helps to prevent the development of a necrotic core due to nutrient deficiency of cells deep within the construct, and provides a readily perfusable network for nutrient perfusion throughout the construct post-implantation. Implantation of an un-vascularized tissue construct requires time for angiogenic infiltration of the host vasculature system into the implant before it becomes fully functional. Within that time, cells within the implant may die off due to lack of nutrients, rendering the implant no longer useful. The implantation of a pre-vascularized tissue construct minimizes the need for vasculogenic and angiogenic processes to occur after implantation and has been shown to induce rapid vascularization upon implantation into mice[36], which provides better long term sustainability of the construct after implantation. Today, success in the vascularization of tissue constructs has been limited to the vascularization of thin (2D) tissue slices, and the vascularization of large 3D tissue

constructs has seen slower progress[37]. The lack of viable fabrication techniques capable of producing in vitro endothelial cords has been a prime hindrance to our progress in this area. Successful vascularization of large 3D tissue constructs would undoubtedly provide invaluable contribution in the field of TE and bring us a step closer to fabricating whole organs.

2.1.3 In vitro models

Besides its obvious applications in regenerative medicine, the ability to vascularize large 3D tissue constructs would also be useful in the development of in vitro tissue models which better replicate our native tissues[38]. The use of animal models and ex-vivo (cadaveric) human tissue models for various studies, such as pathophysiology and pharmacology, has resulted in significant findings over the years. However, there are inherent limitations with the use of these models. Cadaveric human tissue models offer exceptional replication of native tissue, however they suffer from limited availability. The use of animal models brings rise to issues with species-specific tissue response[39], as well as ethical issues with regards to the well-being of lab animals. These shortcomings has driven researchers to develop more advanced in vitro tissue models which accurately mimic native tissue and are easily fabricated without the need for donor tissue and animal experiments such as organs-on-chips[40–42]. The most common in vitro model used in scientific research today is the cell monolayer i.e. 2D cell culture[43]. As we know, the cells in our bodies are enclosed within a 3D ECM where they attach and proliferate. Thus, the results of experiments utilizing 2D in vitro models may not be an accurate representation as it is known that cells function differently when cultured in 2D vs 3D cellular environments, affecting cellular cues (inputs from microenvironment which affect cell functions), differentiation, adhesion, and morphogenesis, among others[44–46]. 3D in vitro models are able to more

closely replicate the cellular environment found in our native tissue, thus providing researchers with more reliable results compared to using 2D in vitro models[47]. However, the fabrication and application of 3D in vitro models are not as trivial as cell monolayers. A common problem faced by the application of 3D in vitro models is the loss of cell viability in long-term culture[48], thus driving research to improve the lifespan of these models through modifications to the technique[49]. Vascularized 3D tissue models have the potential to remain viable for long periods while still able to accurately replicate native vascularized tissue. Cell-cell interactions and signalling plays a vital role in determining cell functionality in vivo. As such, monoculture tissue models may not accurately depict and account for the cellular interactions between parenchymal cells (i.e. hepatocytes and cardiomyocytes) and ECs which occur repeatedly in our organs, given that blood vessels are found throughout our entire body. The development of vascularized 3D tissue models is crucial in this regard[50]. Moreover, vascularized 3D tissue models could also be used to boost our understanding of vascular physiology[51], such as vasculogenesis[52] and angiogenesis[53], developmental biology[54], and the processes involved in vascular pathogenesis which would help to improve treatment of patients suffering from arterial diseases such as thrombosis and atherosclerosis[55]. Perfusable 3D tissue models could also be applied in toxicology, drug screening, and disease modelling[56].

ECs play an active role in the delivery of nutrients through blood perfusion and demonstrate tissue-specific changes in phenotype[57]. For example, effective Central Nervous System (CNS) drug delivery is still a challenging feat today as the Blood-Brain Barrier (BBB) possesses unique characteristics compared to peripheral blood vessels, including tighter cell-cell junctions and unique responses to various signalling factors[58]. Drugs that easily diffuse through the walls of peripheral blood vessels may not show such efficacy when applied to the BBB. Thus, specialized BBB tissue

models need to be developed[59–62]. With this in mind, a flexible fabrication technique able to produce vascular models of the various phenotypes present in our vascular system would significantly contribute to the development of this field.

2.2 Current in vitro vascularization approaches

Various in vitro vascularization techniques have been recently reported in the literature including the in vivo approach where perforated, un-vascularized tissue constructs are implanted to allow the host's peripheral vascular system to naturally infiltrate and vascularize the tissue construct[63]. This method requires the timely invasion of the host vasculature into the un-vascularized tissue construct through angiogenic sprouting in order to provide the cells within the tissue construct with adequate nutrients to survive. Naturally, it would take a longer time to vascularize larger tissue constructs by this approach. Thus, it may not be viable for large 3D tissue constructs as the time taken for in vivo vascularization may be too long causing necrosis before a functional vascular network is formed leading to premature failure of the construct. This method also requires poly-surgical procedures to be performed which increases the risk of nosocomial infection. These disadvantages have driven research into the development of in vitro pre-vascularization techniques to fabricate vascularized tissue constructs prior to implantation. The use of in vitro pre-vascularized tissue constructs would speed up the process of anastomosis with host vasculature and provide cells with quick access to a nutrient supply[64]. We will now look at the current methods developed by various research groups to fabricate pre-vascularized tissue constructs in vitro, and discuss their advantages as well as disadvantages.

2.2.1 Bioprinting

The term bioprinting refers to any additive manufacturing technique which uses biological ink to produce living tissue constructs for a variety of applications including regenerative medicine and cellular studies[65], and has been applied to the fabrication of vascularized tissue in vitro[66]. There are numerous bioprinting techniques which rely on fundamentally different principles of fabrication such as extrusion, ink-jet, and laser-based approaches [67]. Bioprinting technology has been a hot topic of research in recent years, given its potential advantages over other conventional techniques, with research groups striving to improve the performance of existing bioprinters[68], developing unique bioprinting technologies[69,70], and expanding the selection of compatible bioinks[71,72]. This pursuit has given rise to novel bioprinting technologies in recent years such as the development of the “freeform reversible embedding of suspended hydrogels” process, able to produce 3D constructs with complex architecture not achievable by conventional approaches[73]. Today, advanced bioprinters with state-of-the-art features such as precise temperature and viscosity control are now commercially available in the market, and researchers have been utilizing these bioprinters to produce significant research. Researchers have demonstrated the ability of bioprinting technology to fabricate hybrid constructs made of multiple hydrogel materials and cell types, offering control of the construct’s mechanical stiffness and composition[74]. Scaffold-free, large diameter tubular tissue constructs have also been produced by bioprinting for vascular TE applications using an indirect agarose molding technique[75]. The technique offers control of the tube’s shape, dimension, and hierarchical branching. The same approach was utilized to fabricate fused toroid-shaped, scaffold-free tissue from an alginate-based mold produced by bioprinting, showcasing the ability to produce viable tissue with customizable architecture[76].

Novel laser-based bioprinting approaches have also been developed in recent years including the Laser-Induced-Forward-Transfer (LIFT) technique and stereolithography (SLA). The LIFT technique involves the focusing of a high powered laser beam onto a photo-absorbent material coated with biological ink. When the photo-absorbent material is exposed to sufficient laser intensity it vaporizes and causes a high-pressure zone which propels a small volume of biological ink onto a donor slide where the ink is collected. By controlling the laser intensity and axial motion, high resolution patterns of biological material can be printed[78,79]. Stereolithography was patented in the 1980's but only recently has the technology found applications in the field of TE as researchers demonstrated its ability to be used for cell encapsulation and the fabrication of 3D tissue scaffolds. Projection stereolithography (PSL) has been utilized to fabricate living tissue constructs with controllable, porous architecture and demonstrated that cell viability was improved due to enhanced nutrient delivery within the porous scaffolds compared to solid scaffolds[80]. Using a Digital Micromirror Device (DMD), pre-vascularized tissues of multiple cell types with complex architecture were produced using a technique similar to PSL[81]. Commercially available SLA systems have been modified to improve and expand the system capabilities for TE applications such as the ability to fabricate 3D tissue constructs comprising distinct layers of different cell types and material composition, thus improving the long-term viability of encapsulated cells[82]. SLA has also been used to fabricate 3D culture chips containing biofunctionalized perfusable networks [83].

The bioprinting approach has also shown potential applications in the field of vascularization of tissue constructs. A key advantage of using bioprinting technology is the ability to fabricate truly three-dimensional microchannel networks which are

perfusable and can be lined with ECs. These 3D networks can be fabricated into pre-designed patterns which could be useful in studying the effects of vascular network spatial organization. Using a newly developed extrusion-based bioprinting approach, 3D tissue constructs consisting of multiple cell types were successfully produced and Human Umbilical Vein Endothelial Cells (HUVECs) were observed to line the lumen of embedded microchannels simulating perfusable blood vessels[84]. Microchannel networks were incorporated into the bulk ECM through the bioprinting of fugitive ink which was later removed, leaving behind microchannels which were then seeded with HUVECs. A similar study, using the same principles but slightly different methodology, was carried out where researchers were able to incorporate an interconnected vascular network within bulk hydrogel containing hepatocytes and showed that perfusion of the vascular network with cell medium was able to sustain metabolic activity of the surrounding hepatocytes[77] (Figure 2.2). Recently, a technique capable of printing cell-laden tubular hydrogel constructs was developed using a multilayered coaxial extrusion system[85]. The technique demonstrated high cell viability, tunable tube dimensions, perfusability, and complex architecture.

Figure 2.2. 3D printed carbohydrate-glass lattice used as sacrificial template in engineered tissues containing living cells to generate interconnected networks lined with ECs and perfused with blood. Scale bars: (B, D, C top-view) 1mm, (C side-view, E) 200μm. (Adopted from ref.[77])

Despite its obvious advantages, bioprinting does suffer from several drawbacks when applied to vascularization of tissue constructs. Firstly, potential problems arising from the use of fugitive ink include the biocompatibility of the fugitive ink, as well as the removal process. Many reports found in literature today utilize the same fugitive ink approach to tackle the problem of vascularization[86] (Figure 2.3). Fugitive ink may contain cytotoxic compounds which are detrimental to cell viability and affect cell phenotype. The process of removing the fugitive ink, such as chemical dissolution and heat treatment, may also affect cell phenotype and lead to abnormal cell function and necrosis. Secondly, established 3D printing technologies such as Selective Laser Melting (SLM) [87] and Fused Deposition Modelling (FDM) [88], commonly applied for biomedical applications to produce load-bearing metallic and polymeric structures, and known for their ability to fabricate objects with complex architecture, cannot be

applied in the field of bioprinting. Excessive shear stress, high temperatures, and the use of non-biocompatible materials prevent the printing of live cells using these techniques. In addition, the fact that materials suitable for bioprinting are generally soft and gel-like, featuring high water content to promote for cell adherence and survival, limits the level of structural complexity achieved in the printed tissue constructs.

Figure 2.3. Bioprinted agarose template to fabricate microchannel networks within Gelatin Methacryloyl (GelMA) hydrogel. Scale bars: 3mm. (Adopted from ref.[86])

2.2.2 Microfluidics (Lithography)

Microfluidic technology has been gaining popularity in research over the past 2 decades with more and more papers containing the keyword “microfluidic” being published[89]. This technology has found applications in many different fields of research, one of which being the vascularization of tissue constructs. Today, advanced lithographic technology allows us to fabricate complex microfluidic networks with ultra-high resolution, giving the user superb control over the networks’ geometrical features. Its small scale minimizes the amount of consumables needed (such as cell

medium) for each experimental run thus reducing cost and increasing throughput. Microfluidic technology has been used in various ways to achieve vascularization [90]. In one approach, microchannel networks were produced within bulk collagen matrix and seeded with HUVECs to simulate perfusable blood vessels[91] (Figure 2.4). The biofunctionality of the fabricated in vitro vessels was demonstrated including HUVEC interaction with pericytes which affected barrier function. In another approach, microfluidic channels were fabricated within bulk agarose hydrogel encapsulating murine fibroblasts. The microfluidic channels were not seeded with ECs but murine fibroblast viability was shown to improve by the perfusion of medium thorough the microchannel networks[92].

Figure 2.4. Microfluidic technology used to engineer microvascular networks within 3D tissue scaffolds for applications in vascular tissue modelling. Scale bars: 100μm. (Adopted from ref.[91])

Another commonly used and exciting approach today involves the encapsulation of ECs within bulk hydrogel where they spontaneously self-assemble into perfusable

vascular networks. Microfluidic technology is used in this method to fabricate the device, as well as to provide the encapsulated cells with medium and supplement perfusion with controlled parameters such as flow rate, flow direction, and pressure. Various microfluidic designs have been developed to suit the objectives of each research project including the replication of dynamic angiogenesis in vitro[93], the creation of a perfusable vascular network on a chip[94] (Figure 2.5) under physiologically relevant shear rates[95], the vascularization of cardiac tissue for improved functionality[96], and the controlled formation and characterization of capillary networks using a microfluidic device[97]. In these studies, directed angiogenic sprouting has been achieved and strong barrier function, as well as perfusable network interconnectivity has been demonstrated. The advantages of this approach include that it has high throughput, and the vascular networks are formed through natural vasculogenic and angiogenic processes which rely on self-assembly of the ECs, allowing the ECs to degrade and migrate through their surrounding ECM freely which is beneficial compared to mechanically constraining the ECs into their lumenized structures[98]. The microfluidic approach also allows for effective real-time visualization of vascular processes post fabrication [99].

However, the vascular networks formed using this approach are limited to a thin planar tissue layer (up to a few hundred microns in height), unlike the networks formed using the bioprinting approach, and may not accurately recapitulate our native vascular networks which are arranged in 3D. Another limitation would be that the formed vascular networks are confined within the microfluidic system and cannot be easily separated for implantation, thus its application for regenerative medicine is diminished.

Figure 2.5. Microfluidic technology used to form perfusable 3D vascular networks along with tumor vasculature by the spatially controlled co-culture of ECs with stromal fibroblasts, pericytes or cancer cells. Scale bars: 100μm. (Adopted from ref.[94])

2.2.3 Micropatterning

The micro-patterning approach to vascularization involves the patterning of biological material or adhesive proteins on a substrate to induce directed vasculogenesis with controlled spatial organization[100,101]. In one study, researchers used standard photolithographic techniques to produce Polydimethylsiloxane (PDMS) stamps, which were subsequently used to pattern Fibronectin (Fn) strips on glass coverslips following the procedure shown in Figure 2.6(a)[102]. Human Endothelial Progenitor Cells (hEPCs) showed preferential adhesion on the Fn surface compared to the non-adhesive PEG surface while also demonstrating directed elongation along the Fn strips after 24 hours post seeding Figure 2.6(b). Optimal strip width for directed cell elongation was found to be 50μm. After 5 days in culture, immunostaining was performed to show confinement of hEPCs within the Fn strips with sparse migration to neighbouring strips Figure 2.6(c). Another common approach is the use of photolithographic techniques to produce substrates with design-specific

microgrooves which can be filled with cellular material and cultured in vitro. Raghavan et al. utilized this technique in their work which successfully produced lumenized vascular tubes with controlled diameters by varying the dimensions of their microgrooves. By culturing cellular material within branched microgrooves with varying designs, lumenized vascular tubes were also observed to branch into multiple tubes while maintaining their lumenized structure[103]. The branching patterns could be controlled by fabricating microgrooved structures with different designs. Using a similar technique, Chaturvedi et al. developed a technique to successfully produce vascular tubes within microgrooved structures which could be harvested and encapsulated within bulk fibrin hydrogel to produce vascularized tissue used for in vivo implantation to study the impact of various design parameters on the vascularization of TE constructs upon implantation in rats[104]. This is an advantage over the closed microfluidic systems where vascularized tissue could not be harvested for subsequent in vivo implantation. However, the harvesting process needs to be further developed to increase throughput and achieve 3D vascularized tissue. Photolithographic techniques have also been used directly to pattern photo-crosslinkable cellular material onto adhesive substrates for vascularization applications. In one study, a UV source, a photomask, and photo-crosslinkable hydrogel called Gelatine Methacryloyl (GelMA) were used to pattern cell-laden GelMA strips, containing ECs and other cells self-aligning cells, onto treated glass slides to demonstrate the ability to control cell alignment and elongation orientation by mechanically confining the cells within a 3D architecture[105]. In another study using a similar approach, strips of GelMA micro-constructs containing ECs and of varying dimensions were patterned onto a treated glass slide where after culture endothelial tubes formed within the patterned strips [106]. They found that optimal tube formation was only achieved at a given micro-construct size. A variety of other

micropatterning techniques have also been used for vascularization applications such soft lithography[107] and laser-assisted micropatterning[108].

In all the aforementioned studies, successful engineering of endothelial tubes were reported with controlled spatial organization. However, similar to the microfluidics approach, micropatterning is only capable of producing patterned cellular material on a planar 2D surface, which fails to recapitulate the 3D nature of native tissues.

Figure 2.6. PDMS stamping technique used for Fn micropatterning on coverslips. hEPCs were confined within Fn strips and demonstrated controlled elongation along strip direction. Scale bars are 50μm. (Adopted from ref.[102])

2.2.4 Wire molding

The incorporation of microchannels within a TE construct allows immediate perfusion of medium throughout the tissue construct to supply cells with adequate nutrients for survival. The wire molding technique is a simple and effective method of producing microchannels within a tissue construct which can be perfused with medium to enhance the viability of cells within the surrounding polymerized gel[109]. The fabrication procedure used previously by another research group is shown schematically in Figure 2.7[110]. A pre-polymer solution (with or without cells) is cast and polymerized around a wire held in suspension by mechanical supports. After complete polymerization, the wire is then manually withdrawn from the polymerized material leaving behind a perfusable microchannel which provides nutrients to encapsulated cells thus simulating our native microvessels. The generation of microporous cell-laden hydrogel constructs through sucrose crystal leaching was shown to enhance diffusivity through the construct and increase viability of encapsulated cells. A clear advantage of this approach is the ability to incorporate evenly distributed microchannels within large tissue constructs in true 3D form where microchannels are stacked on various Z-planes. This was demonstrated by Yao et al. in their work where multichannel (up to 7) collagen conduits were fabricated using this technique to demonstrate the potential of using multichannel nerve guide conduits, instead of commercially available single channel conduits, to minimize dispersion of regenerating axons[111]. The same approach was utilized for vascularization applications where ECs were seeded onto the inner walls of microchannels within their tissue construct, and after implantation in vivo, demonstrated quicker vascular infiltration compared to tissue constructs without microchannels[63]. In a different paper, Chrobak et al. used the wire molding technique to create a single microchannel within bulk collagen gel, after which ECs were seeded and grown to confluence on the

inner walls of the microchannel[112]. They used a sharply tapered needle to produce a single channel with varying diameters along its length (75-120 μ m). The relationship between channel diameter and gelling temperature was established, as well as the relationship between EC invasion into the surrounding matrix and collagen concentration. The vascular tubes also demonstrated appropriate response to inflammatory stimuli such as Histamine and Thrombin, which showed that they were functioning as native vessels would in vivo. Our native vessels are composed of more complex architectures than just a monolayer of ECs. Capillaries mostly comprise a bilayer structure of ECs surrounded by a Smooth Muscle Cell (SMC) layer responsible for defining vascular tone. By utilizing a modified wire molding technique in combination with other unique procedures, the ability to recapitulate this bilayer structure in vitro was demonstrated in recent reports, specifically using SAM-based cell transfer mechanisms[113] and hierarchal cell manipulation techniques[114]. In a separate study, a cell-seeded microchannel fabricated by wire molding was used to determine the impact of mechanical signals on the stability and barrier function of engineered microvasculature simulating native vessels[115]. These works show the variety of research applications where the wire molding technique could be used to help us increase our understanding of vascular biology through the use of in vitro models.

Figure 2.7. Wire molding technique employed to fabricate perfusable 3D microvascular tubes within microporous cell-laden hydrogels to produce biomimetic tissue constructs. (Adopted from ref.[110])

Wire molding has proven to be a promising technique for the vascularization of large 3D tissue constructs. It is a simple technique and offers immediate perfusability as well as precise control over microvascular diameter. The channel diameters referenced in this chapter range from as small as 55 μm [112] to 620 μm [114], demonstrating the wide diameter range achievable using this technique. Endothelial layers seeded onto microchannel walls demonstrated healthy phenotype similar to our native vessels. The ability to vascularize thick 3D constructs could also be advantageous over other techniques which are confined to thin sheets of tissue.

However, the design complexity of the fabricated microchannel networks are limited to simple designs and cannot be precisely controlled using this technique, thus it is unable to effectively replicate the complex architecture of our native vascular networks.

2.2.5 Cell sheet engineering

A novel approach to TE is the use of cell sheet technology, which is conceptually different from the other aforementioned techniques. Cell sheet technology allows the user to harvest confluent cell monolayers from culture dishes with the use of thermo-responsive polymers which allow easy detachment without chemical treatment. The harvested cell sheets remain viable and intact with their naturally deposited ECM which allow for easy reattachment onto another substrate after harvest. Cell sheet engineering has been used to engineer biomimetic tissue in vitro such as corneal epithelium[116], skin[117], and myocardial tissue[118].

Asakawa et al. applied cell sheet technology to fabricate vascularized tissue constructs made of stacked cell sheet layers[119]. EC monoculture sheets were stacked with fibroblast monoculture sheets in multiple different configurations to study the effect of EC positioning within the tissue construct on vascularization. After

3 days in culture, in vitro vascular networks were formed within the cell sheet stacks, and these pre-vascularized constructs showed enhanced vasculogenesis upon implantation in vivo. Subsequent reports managed to control the alignment of endothelial networks through cell-cell interactions with surrounding focally oriented fibroblast sheets[120]. In another study, a thick (30 cell sheets, close to 1mm thick) myocardial tissue stack with an interconnected, perfusable vascular network was fabricated using cell sheet technology in tandem with in vivo vascularization achieved by subcutaneous poly-surgery (up to 10 cycles) implantation into nude rats[121]. This approach may not be feasible for clinical translation as it would require the patient to undergo repetitive surgical procedures. A technique able to vascularize thick cell sheet stacks in vitro would negate the need for poly-surgery. Sakaguchi et al. proposed a strategy for thick cardiac tissue vascularization in vitro using cell sheet technology in combination with a perfusion bioreactor and microfluidics[17,122] (Figure 2.8). Stacks of cell sheets consisting of cardiac and ECs were layered on top of a collagen construct containing microchannels and cultured in a bioreactor. ECs within the cell sheets were seen migrating through the collagen ECM to form vascular networks which were connected to the pre-fabricated perfusable microchannels, thus allowing medium perfusion throughout the layered tissue construct. As more sheets were stacked on the layered construct (12 sheets, more than 100µm in thickness), ECs continued to form new vessels and connect with pre-existing microvessels to form an interconnected vascular network. Using a similar method, Sekine et al. demonstrated in a study that stacked layers of cell sheets composed of neonatal rat cardiomyocytes and ECs improved cardiac function when implanted into infarcted rat hearts with increasing EC density, and showed higher capillary density and inosculation[123].

Figure 2.8. Cell sheet technology combined with a collagen based perfusion bioreactor for the preservation of cell viability by the vascularization of 3D tissues. (Adopted from ref.[122] URL: <http://www.nature.com/articles/srep01316>)

Overall, cell sheet technology offers a unique method for the vascularization of tissue constructs with distinct advantages. Firstly, the ECM material is deposited naturally by the cells themselves, thus negating the need to fabricate a biodegradable scaffold which may require the use of cytotoxic chemicals. Secondly, the high cell-density and homogeneous distribution achieved in a cell sheet leads to higher regenerative function[17], and the method of cell sheet harvesting and stacking only requires slight thermal treatment which does not significantly harm the cells. Thirdly, the ability to form 3D vascularized tissue consisting of several cell layers and control over the orientation of vascular networks has been demonstrated using cell sheet technology.

The main downside to this technique is that the process tedious, and the tissue takes a long time to develop. The level of control over vascular network architecture is also low compared to the previously mentioned techniques like micropatterning and microfluidics.

2.2.6 Summary and comparison

In vitro vascularization techniques play a critical role in the advancement of TE. In the field of regenerative medicine, scientists have identified vascularization as a key hurdle that needs to be overcome. To date, the variety of tissue-engineered products successfully translated for clinical use has been limited to thin avascular tissue due to the inability of current technology to incorporate functional vascular networks into thick tissue constructs. The ability to fabricate physiologically accurate in vitro tissue models has also been hindered by the lack of effective in vitro vascularization techniques. Although 2D vascular models have been successfully fabricated and proven their efficacy, thick 3D vascular models remain elusive. Today, biologists and engineers are working hand in hand to develop working techniques for in vitro vascularization. Described in this section are several enabling technologies being developed today which show promising signs of being able to achieve this goal.

Table 2.1 shows a comparison of the capabilities possessed by the 5 techniques covered in this review. Each of these techniques has its own unique capabilities which make it particularly suitable for certain applications, such as for fabricating 3D perfusable networks within a tissue construct (bioprinting, cell sheet engineering), for controlled branching patterns and vessel diameter (micropatterning, wire molding), and for fabricating 2D in vitro vascular models (microfluidics, wire molding). The ability to produce networks with controlled architecture using the bioprinting and micropatterning techniques would be useful for TE applications as well as in vitro vascular models.

There are other approaches being applied to achieve in vitro vascularization such as electrospinning[124], cell-accumulation[125], and vascular corrosion casting[126] which were not covered in this review but also demonstrate potential for future development.

Table 2.1. Comparison of characteristics possessed by the different vascularization techniques.

	Bioprinting	Microfluidics	Micropatterning	Wire molding	Cell-sheet
Vascularization of thick 3D tissue	✓			✓	✓
Control of tube dimensions	✓	✓	✓	✓	
Control of network architecture	✓		✓		
Perfusable networks	✓	✓		✓	✓
Multicellular vascularized tissue	✓	✓	✓	✓	✓
Suitability for in vitro models	✓	✓	✓	✓	
Tubulogenesis through self-assembly		✓	✓		✓
Ability for vascularized tissue to be harvested for downstream experiments	✓			✓	✓

With the increasing flow of research into bioprinting technology, it is not surprising that the technology has experienced a rapid boost in development. Bioprinting technology now allows us to print multicellular constructs with high precision which mimics the hierarchical architecture of native tissue. It also possesses the ability to fabricate perfusable 3D microchannel networks within bulk tissue which is particularly useful in our efforts to achieve in vitro vascularization. The bioprinting technique has the potential to be improved significantly and to find new applications in the years ahead. It appears that bioprinting is a promising technique for the future of TE and could potentially evolve into becoming the gold-standard of biofabrication technology.

2.3 Bessel beams

Lasers have previously been used to cut microchannels within a material for the fabrication of blood vessel-like models through direct/indirect writing techniques[127]. These techniques are different from additive manufacturing techniques where constructs are fabricated by the sequential addition of material instead of subtraction (i.e. material ablation). Laser-based additive manufacturing techniques today use photosensitive resin which polymerize upon exposure to lasers at various wavelengths. Such techniques include Stereolithography (SLA), Projection Stereolithography (PSL), and Digital Light Processing (DLP). The difference between

these techniques is the type of light used for resin exposure. In SLA, a Gaussian beam is focused using multiple lenses into a high intensity voxel which is used for polymerization. The voxel covers a small volumetric area in 3D space and therefore requires translational movement in order to print a single layer. A variant of SLA is the 2-Photon Polymerization (2PP) process which can achieve nanoscale print resolutions using the same general mechanism[128]. PSL and DLP on the other hand expose the resin to light in a pre-designed shape which corresponds to the shape of the layer it is printing through the use of photomasks and Digital Micromirror Devices (DMDs), respectively. In this way, each layer can be printed through a single exposure, resulting in quicker fabrication time but sacrificing print resolution in the process. In this dissertation the use of BBs for use in additive manufacturing, which is unlike any of the previously mentioned techniques, is studied.

BBs have a unique profile, as illustrated in Figure 2.9(a-b), consisting of a bright core surrounded by a series of dark and bright rings[188]. The BB core has the greatest intensity with every subsequent bright ring having reduced intensity. In Figure 2.9(a), the dotted ring encircles the BB core and the first bright ring, which is denoted in Figure 2.9(b). This figure is referenced in subsequent Section 5.3.3.

Figure 2.9. BB intensity profiles. Dotted ring in (a) encircles the BB core and the first bright ring denoted in (b). Adopted from ref. [188].

A BB can be produced through various means, including passing a Gaussian beam through a specially manufactured axicon lens (Figure 2.10a). BBs possess several unique characteristics which make them particularly useful for additive manufacturing applications[129]. BBs possess a highly localized, high intensity, non-diffracting core with an extended depth of field within its non-diffracting region (Figure 2.10b) compared to a focussed Gaussian Beam (Figure 2.10c)[130]. This high intensity BB core is the result of constructive interference of the beam after exiting the axicon. Unlike focused Gaussian beams, the high intensity BB core extends throughout the length of the non-diffracting zone while retaining its highly localized profile. Thus by exposing a pre-polymer solution to the BB core, a long fiber-like structure can be produced through a single exposure as the high intensity BB core propagates through the entire height of the solution.

Figure 2.10. (a) Using an Axicon lens to produce a BB from an incident Gaussian Beam. (b) BB profile with extended depth of field, showcasing high intensity, non-diffracting core. (c) Focussed Gaussian Beam profile showing small voxel of high intensity, as compared to BB profile. (Adopted from ref.[130])

These unique characteristics of BBs has led to their use for novel applications including microfabrication of transparent dielectrics[132], high aspect ratio microdrilling through both transparent[133] and opaque[134] materials, optical manipulation of micro-particles through the optical tweezer effect[131,135–137], and the fabrication of microscale constructs for biological applications[138]. Researchers have also used BBs to fabricate high resolution microstructures using the 2PP technique which is based on photo-polymerization[139–141]. 3D polymeric scaffolds

with sub-micron features have also been fabricated by translating the BB core in a controlled grid pattern within a pre-polymer resin[142,143]. Exploiting the extended depth of field of the BB core, entire 3D structures were made by single scan parallel processing, negating the need for the layer-by-layer scanning required in SLA and thus reducing processing time. Polymeric fibers[145] and structures with more complex architectures[144] have also been fabricated using BBs. The distinct advantages of using BBs for additive manufacturing make it suitable to be applied in the field of TE where high-resolution fabrication techniques with short fabrication times are in demand. However, there is a lack of reports in the literature that study the use of BBs for the fabrication of TE constructs. Hydrogels are a commonly used material in the field of TE due to its high water content and microporous structure which provides a conducive environment for cell growth[228-229]. So far, the use of BBs to fabricate hydrogel constructs with customizable design has not been reported in the literature. More so, the use of BBs for the direct printing of live cells has also not been reported.

2.4 Endothelial cords

One of the objectives of Chapter 5 in this dissertation is to utilize the BB technique for TE applications, specifically, for the successful fabrication of endothelial cords in vitro. This section will explain what endothelial cords are, the significance of fabricating endothelial cords, in vitro, and the current state of research from the literature.

In this dissertation, a “tube” is defined as a perfusable lumenized structure that has been demonstrated to be hollow. A “cord” on the other hand, is defined as a structure that mimics the circular cross-section of a tube, but has not been demonstrated to be truly hollow and perfusable. While this terminology has not been followed in the current literature, as there are reports that claim to have fabricated “tubes” that have not been shown to be truly hollow and perfusable, it will be used in

this dissertation to provide clarity to the reader and to avoid misrepresentation of the data. An “endothelial cord” is a cord that is made up of ECs.

Our blood vessels are basically lumenized, perfusable tubes that carry blood and nutrients to cells located all around our bodies. Although research into the fabrication of in vitro lumenized endothelial tubes that mimic our native blood vessel architecture has seen limited success, research groups have been able to successfully produce in vitro endothelial cords, which can be considered a primitive blood vessel. From the literature, reports have suggested that the formation of endothelial cords precedes the formation of mature vasculature[106]. It has been shown that incorporating primitive vascular networks within TE grafts enhanced anastomosis of the “pre-vascularized” graft with the host vascular system post-implantation [257-259]. Cord diameters were effectively controlled through manipulation of the fabrication parameters and hydrogel concentration[103], which was shown to affect the density and position of the resulting vascular network post-implantation into nude mice[104]. Implanted endothelial cords into mouse models was also shown to induce capillary formation along its length which were perfused with blood by day 3 post-implantation[36]. Thus the ability to fabricate endothelial cords in vitro would contribute significantly in the field of TE and pre-vascularization strategies.

From the literature, research groups have predominantly used photolithographic techniques to encapsulate HUVECs into hydrogel channels which then self-assemble into endothelial cords. The general procedure and mechanism is illustrated in Figure 2.11(a). To demonstrate that endothelial cords have been successfully fabricated, most groups have utilized staining techniques combined with confocal microscopy to capture images of the cords and their cross-sections[103,106]. If cross-sectional images show that the HUVECs arranged themselves into cord structures with circular

cross-sections, then it is deemed to be a successfully fabricated endothelial cord (Figure 2.11b-c). In this dissertation, the confirmation of endothelial cord formation will also be based on this method of characterization as shown in Section 5.3.5 of Chapter 5.

Figure 2.11. Formation and characterization of endothelial cords. (a) Fabrication procedure using photolithographic techniques to produce micro-grooved templates which are filled with cell-laden collagen gel. (b) Phase-contrast images of endothelial cords. (c) Confocal images of endothelial cords showing circular cross-sections. (Adopted from ref.[103])

3. Laser-based 3D printing using Bessel Beams

Work presented in this chapter is largely based on the following publication: **Liew, Wen Loong Andy, and Yilei Zhang. "Laser-based fabrication of 3D hydrogel constructs using bessel beams." *Bioprinting* (2018) 9 44-51.**

3.1 Introduction

Successfully fabricated TE constructs can be applied in the field of regenerative medicine where the construct is implanted into a patient's defect site to replace or restore damaged tissue. TE constructs are also used for in vitro studies to further our knowledge in areas of developmental biology[54], pathology[146], toxicology[56], drug-screening[147], and others.

TE constructs can be classified into 2 general categories based on their fabrication approach: (1) Scaffold-based constructs (2) Scaffold-free constructs. In scaffold-based constructs, cells are either seeded on the surface of a pre-fabricated scaffold or directly encapsulated within the scaffold material itself. In scaffold-free constructs, cells are cultured without a pre-fabricated scaffold and harvested, resulting in tissue with high cell density. Although scaffold-free constructs such as cell-spheroids[148] and cell sheet engineering[122,149] have shown promising results, scaffold-based constructs still remain the dominant approach due to several advantages. The scaffold serves as a man-made ECM and provides structural integrity for the tissue construct. It also provides a platform for cell adhesion and a conducive environment for cell growth which mimics our native tissue. Scaffolds with interconnected microchannels have a high surface area to volume ratio which is desirable in TE as it increases nutrient exchange between cells and their surroundings. It also allows the controlled release of desired proteins and growth factors to the attached cells. Numerous scaffold fabrication techniques have been developed over the years and are still being

developed today. These techniques include particulate leaching[150], freeze-drying[151], gas foaming[152], electrospinning[153], and additive manufacturing[81]; the latter 2 techniques being more recently developed.

Additive manufacturing or 3D printing techniques in the field of TE involves the layer-by-layer build-up of biomaterial based on extrusion[154], inkjet printing[155], or photopolymerization[156]. Acellular printed constructs are typically made of biocompatible materials which require subsequent cell-seeding while the direct printing of cellular material results in a scaffold with cells already encapsulated within the scaffold material itself. Both these approaches have been used to successfully engineer viable tissue in vitro. Bioprinting technology offers several unique advantages compared to other approaches. It allows the fabrication of TE constructs with unique, complex architecture to suit patient-specific requirements. Cellular material can be deposited in pre-designed patterns for in vitro tissue research. Developments in the printing of sacrificial templates have led to the fabrication of customizable microchannel networks within 3D tissue constructs[77]. However there are also shortcomings to the bioprinting approach. Long processing times are common for larger constructs due to the layer-by-layer build-up. Due to the bottom-up fabrication approach where material is added on instead of subtracted away (i.e. material ablation), many conventional printing techniques rely on the concurrent fabrication of support structures to prevent structural collapse of the construct during printing of high aspect ratio structures i.e. tubular constructs [75,157,158]. In addition, there is also room for improvement in print resolution to increase the design intricacy of printed constructs to better replicate the native environment.

This chapter describes a laser-based printing technique using BBs for the fabrication of 3D hydrogel constructs. All equipment, methods, and materials used for printing

are detailed for reproducibility. The technique is used to fabricate customizable, acellular scaffolds and tubular constructs made of hydrogels which is a material commonly used in TE due to its biomimetic properties. The advantages and limitations of the BB printing technique are discussed as well as how the technique is capable of addressing the shortcomings of existing printing technology as previously described.

This chapter does not include the use of live cellular material for printing as its main focus is to give the reader a fundamental understanding of how the technique works and how it compares to other printing techniques in terms of printing time, printable constructs, and print resolution. The study on the cytocompatibility of the printing technique is covered in Chapter 5.

3.2 Methods and materials

3.2.1 Optical setup

A schematic diagram of the optical set-up used for fabrication is shown in Figure 3.1(a). A UV laser source (355nm) produces a Gaussian beam (solid blue lines) which propagates through a series of lenses, mirrors, and an aperture. Propagation through the Axicon lens ($\alpha=179^\circ$, Thorlabs) produces the BB denoted by the dotted red line. A vat filled with pre-polymer solution was placed on a translation stage with programmable translation motion and positioned within the propagation axis of the BB for crosslinking. A photo of the actual set-up used is shown in Figure 3.1(b) with the cyan arrows denoting the Gaussian beam and the red arrows denoting the BB formed after the Axicon.

Figure 3.1. (a) Schematic diagram of optical set-up, laser propagation, and manipulation. Dotted red line indicates the BB. (b) Actual experimental set-up. Cyan arrows indicate the Gaussian beam while red arrows indicate the BB.

3.2.2 Pre-polymer preparation

3.2.2.1 Gelatin Methacryloyl (GelMA) preparation

Lyophilized GelMA was taken from -80°C storage and added to DPBS at 5%w/v and left to dissolve at 60°C for 3 hours. 2-hydroxy-1(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone photoinitiator (Irgacure 2959, CIBA Chemicals) was added at 0.5%w/v to the GelMA solution and left to dissolve completely at 60°C for 1 hour. The concentrations of GelMA (5%w/v) and photoinitiator (0.5%w/v) used are based on current literature where these concentrations are commonly applied for tissue engineering studies[109,160,174,178,180].

3.2.2.2 PEGDMA preparation

Poly(ethylene glycol) dimethacrylate (PEGDMA, Mn 750, Sigma) was added to DI water at 10%, 30%, and 50%(w/v) and left to dissolve at 60°C for 1 hour. The concentrations of PEGDMA used for testing were selected based on previously tested concentrations in the literature which range from 10% to 80% concentration[162]. Irgacure 2959 photoinitiator was added at a constant concentration of 0.5%w/v to the each PEGDMA solution and left to dissolve completely at 60°C for 1 hour. 30% PEGDMA gels were used for the fabrication of all constructs in Section 3.3. 10% and 50% gels were used only for mechanical characterization. The factors that affect the mechanical properties of photocrosslinkable gels include gel concentration, degree of functionalization (i.e. methacrylation), concentration of photoinitiator, gel composition, and degree of crosslinking. In this dissertation, the effects of gel concentration, degree of functionalization (in Section 5.3.2), and gel composition (in Section 5.3.2) are studied. However, it should be noted that the concentration of photoinitiator used and the degree of crosslinking may also affect the mechanical properties of the crosslinked gel.

3.2.3 PEGDMA mechanical testing

Compression tests were performed at room temperature on PEGDMA samples according to previously published procedures[86,109,159] with slight variations when compared to these procedures including compression rate (1 mm/min[86], 0.1 mm/min[159]), sample height (750 μ m[109], 1 mm[159]), and mechanical tester used (Instron 5542[86,109,159]). PEGDMA pre-polymer solution was prepared at varying concentrations of 10%, 30%, and 50%(w/v) in DI water. Cylindrical samples were prepared by pipetting 80 μ L of solution into an 8mm diameter mold and exposing the solutions to UV light (UVF400/600 UV Flood System, 250-650 nm, 50% power setting, 120 s) for crosslinking. The resultant gel samples were 1.6mm in height and retained

their 8 mm diameter and cylindrical shape after removal from the mold. 3 samples were prepared for each concentration. After UV exposure, the samples were removed from the molds and soaked overnight in DI water to ensure complete swelling before mechanical testing. Samples were blotted lightly with Kimtech wipes to remove excess water before being loaded onto an Instron 5566 mechanical tester for compression loading using a 100N load cell. Samples were compressed at a rate of 20% strain/min. Compressive modulus was determined based on the slope of the stress-strain curve.

3.2.4 Fabrication process

3.2.4.1 Printing of hydrogel constructs

The fabrication process is shown schematically in Figure 3.2. Printing was carried out at room temperature. First, the vat filled with pre-polymer solution (GelMA or PEGDMA) is placed on the translation stage and the stage is centered along the BB propagation axis. Static exposure of the pre-polymer solution to the BB (red line) results in localized crosslinking (blue line) and gel formation throughout the entire height of the pre-polymer solution. Translational motion of the stage coupled with BB exposure results in the crosslinking of 3D hydrogel constructs. Print settings were optimized prior to construct fabrication and kept constant for all 3D constructs: Laser power = 120 μ W, Magnification (M) = 1 (refer to Section 5.2.2), translational speed of stage = 1mm/s.

Figure 3.2. Mechanism for the fabrication of hydrogel constructs. Exposure of the pre-polymer solution to the BB results in localized crosslinking. Translational stage motion coupled with BB exposure results in the crosslinking of customizable hydrogel constructs.

3.3 Results and Discussion

3.3.1 Fabrication of customizable 3D constructs

Customizability

Tubular constructs are used in the field of TE to produce substitutes for tubular organs such as trachea[21], esophagus[23], lymph vessels[171], vascular grafts[172,230] and Nerve Guide Conduits (NGCs)[173,231]. These tubular constructs vary in size and shape depending on the anatomical structure of the target organ it is meant to restore. For example, decellularized tissue engineered vascular grafts[230] and electrospun NGCs[231] had cross-sectional diameters that varied significantly in size at 16 mm and 2 mm, respectively - an approximately 8 fold difference.

Using the BB printing technique, 3D constructs with customizable cross-sections were fabricated as shown in Figure 3.3(a) by varying the translation profile of the stage, demonstrating the flexibility to customize cross-sectional shape and size of the constructs. The resultant cross-section of the construct is maintained along the construct height and is visually comparable to the pre-designed translation profile.

Tissue engineered tubular constructs also come in different lengths designed to suit patient-specific requirements, for instance, patients suffering from more serious injuries may require longer tubular grafts than others. The height of the pre-polymer solution in the vat was controlled at 2 mm and 6 mm (refer to Figure 3.2) and two constructs with identical print parameters and translational profile were printed. The printed constructs had heights of 2 mm and 6 mm, respectively, following the heights of the pre-polymer solution in the vat during fabrication (Figure 3.3b). In this way, the heights of the fabricated constructs could be accurately control.

Figure 3.3. Fabrication of customizable 3D constructs. **(a)** 3D PEGDMA constructs with 'window' and 'square' cross-sections. **(b)** Controllable construct height (2mm and 6mm shown) depending on the height of the pre-polymer solution in the vat during printing. **(c)** Fabricated perfusable high aspect ratio tubular construct with 'square' cross-section; 1cm height and inner-wall length 2mm. **(d)** Thick-walled (top) and thin-walled (bottom) tubular constructs with translational profiles shown in the insets.

Vascular grafts

Synthetic large diameter vascular grafts are commercially available and are being used today for arterial bypass surgery[232]. However, small diameter vascular grafts (below 6 mm) aimed at replacing damaged blood vessels have not seen the same

level of success due to high occlusion rates post implantation resulting in poor performance[233]. Today, autologous arterial grafts are harvested and used for small diameter vascular replacement, however, this requires multiple surgical procedures and leads to increased risk of nosocomial infection, and the number of grafts available to be harvested from a patient is also limited[236]. Thus, there is a need for the development of synthetic small diameter vascular grafts that exhibit comparable efficacy to their large diameter counterparts. Our native blood vessels vary significantly in diameter and wall thickness, from capillaries (8 μm) to arteries (8 mm), and everything in between. A flexible fabrication technique capable of producing tubular constructs with anatomical relevance and customizable dimensions to suit user requirements would be significant in the pursuit of small diameter vascular graft development.

Research into the development of small diameter vascular grafts has produced a wide range of tubular constructs in terms of construct dimensions. From smaller synthetic grafts with inner lumen diameter of 500-600 μm , 150 μm wall thickness, and 3mm length[234], to larger grafts with inner lumen diameter of 4.75 mm, 0.3 mm wall thickness, and 4 cm length[235]. Using the BB printing technique, tubular constructs were fabricated with dimensions that were easily controllable by varying the translational profile of the stage. To demonstrate this capability, tubular constructs were fabricated with anatomically relevant dimensions in between the aforementioned examples from the literature. The tubular constructs fabricated using the BB technique had a 2 x 2 mm cross-section with patent lumen, as demonstrated by passing a needle tip unobstructed through the lumen of the construct in Figure 3.3(c), with thick or thin walls (0.5 mm and 200 μm , respectively, Figure 3.3d), and 1 cm length. The construct of 1 cm length was produced by increasing the vat height of

the pre-polymer solution to 1 cm. Wall thickness was controlled by increasing the number of scan lines in the translation profile as shown in the inset.

The suturability of the hydrogel conduits fabricated in this dissertation to a host model was not tested. However, from the literature, there are numerous research papers investigating the capabilities of various hydrogel conduits for vascular grafting applications. To name a few, materials tested include Ca alginate/polyacrylamide hydrogel with braided fiber strut[260], polyacrylamide hydrogel sandwiched between layers of braided silk and polyurethane[261], chitosan-based hydrogel[262], and gelatin-alginate methacryloyl (GEAL) with PCL fibers[263] demonstrating promising results for use as synthetic vascular grafts in terms of mechanical properties and biocompatibility. The suture strength of grafts made from GEAL were reportedly similar to human internal arteries, and demonstrated suture retention in in vivo rabbit models. In this dissertation, fiber reinforced or composite hydrogel tubes were not fabricated using the BB printing technique and should be evaluated in future work in light of these reports.

Fabrication time

For printing techniques used in tissue engineering applications for the direct printing of live cells, fabrication time is an important criterion. The longer the processing time, the more time the cells are not exposed to a conducive growth environment (i.e. growth media and appropriate incubation temperature and humidity) thus resulting in lower cell survival. On the other hand, a rapid printing technique would be capable of fabricating tissue engineered constructs in a fast and efficient manner, ensuring the timely treatment of patients and reducing operating cost in the long run. Ideally, printing techniques used for tissue engineering applications should have short fabrication times, however, existing printing technologies rely on the layer-by-layer

build up of material which is often time consuming as each layer is printed individually. Using the BB printing technique, where the entire tubular construct was fabricated without layer-by-layer processing, the fabrication time was significantly reduced compared to existing bioprinting techniques. For comparison, a detailed study on the feasibility of fabricating vertical alginate hydrogel tubular constructs was published using an extrusion-based system[163]. Based on this report - neglecting extrusion time, cross-linking time with CaCl_2 solution, and processing time between printed layers - the approximate fabrication time was no less than 50 seconds to build a construct similar to the one shown in Figure 3.3(c). Using the BB technique, the average fabrication time was reduced to 20 seconds, which is a reduction of more than half.

Aspect ratio

The fabrication of high aspect ratio, thin-walled tubular structures using existing 3D printing techniques, such as those shown in Figure 3.3(c), poses a challenge due to structural instability of the construct during printing which may cause it to collapse. This occurs when the first few deposited layers are unable to support the weight of the subsequent printed layers due to insufficient crosslinking, common for techniques with crosslinking mechanisms which take a longer time such as extrusion-based chemical crosslinking techniques. As a result, existing printing techniques today rely on the concurrent fabrication of support structures surrounding the construct itself to provide the structural integrity necessary for successful printing. Printing techniques using scaffold-free cell aggregates[75,157] and methacrylated hyaluronic acid (HA-MA)[158] as print material required the concurrent fabrication of supporting hydrogel structures, which added to the fabrication process complexity, materials required, and time required due to the subsequent support material removal procedure. Using

the BB technique, it was demonstrated that no support material was necessary to produce the high aspect ratio tubular constructs.

Other applications

Although the focus of this dissertation focuses on the applications of the BB technique in vascular tissue engineering, there are other areas in the field of tissue engineering where the BB technique may be applied. PEG-based hydrogels, such as PEGDMA used for printing in this chapter, are commonly used in the field of TE due to their biocompatibility, their ability to be treated and modified, and their mechanical strength. Articular cartilage, according to literature, has compression modulus of between 0.08-2.1MPa from the superficial to deep layers of adult bovine cartilage[162]. Results of the mechanical characterization of PEGDMA samples used in this chapter reveals comparable mechanical properties at 30% and 50% gel concentration - the compressive modulus for 30% and 50% gels were 0.1MPa and 0.38MPa, respectively (Figure 3.4). Thus, the mechanical properties are shown to be tunable and comparable to articular cartilage, making it suitable for future applications in articular cartilage regeneration. However, it should be noted that mechanical properties alone do not fully justify the applicability of a certain material in tissue engineering as there are many other factors to consider such as biodegradability, cytotoxicity, and porosity among others.

Figure 3.4. Mechanical properties of PEGDMA gel. **(a)** Compressive stress-strain curve of 10% (blue), 30% (green), and 50% (red) PEGDMA gels. **(b)** Compressive modulus of PEGDMA gels at varying concentrations. Error bars represent SEM (* $p < 0.01$, $n = 3$).

3.3.2 Fluorescent bead encapsulation within tubular constructs

In native tissue, cells attach themselves onto the 3D ECM in which they are encapsulated. It is known that the 3D encapsulation of cells within hydrogel scaffolds has significant advantages over 2D culture as it more accurately mimics the in vivo environment which improves cell-cell contacts, cell-matrix interaction, and induces more natural cell phenotype i.e. differentiation[44]. Thus, a fabrication technique capable of direct cell encapsulation within the hydrogel material itself would be beneficial for TE applications.

In this section, fluorescent micro-beads (10 μm) were encapsulated within 3D hydrogel constructs to simulate the encapsulation of cells using the same technique and the same procedure as in Section 3.3.1, except that fluorescent beads were suspended in the pre-polymer solution prior to printing. Figure 3.5(a) shows a fluorescent bead-laden tubular construct fabricated using this technique. When exposed to light of the appropriate wavelength, particles are clearly seen at a high density and evenly distributed within the construct. Fabrication of fluorescent bead-laden constructs with customizable cross-sections (Figure 3.5b; triangle cross-section) and control of wall thickness (Figure 3.5c) was also demonstrated. However, there was more pronounced deformation of particle-laden constructs compared to particle-free constructs, thus indicating that the presence of opaque particles was responsible for the deformation. Figure 3.5(d) shows the deformation at the bottom of the constructs with triangular and square cross-sections. Wall thickness decreased gradually toward the bottom of the constructs resulting in the deformation. The magnified inset image in Figure 3.5(b) shows the wall thickness decreasing effect.

Wall thickness at the top and bottom of the construct are indicated by white arrows showing a gradual decrease in wall thickness toward the bottom of the construct. Wall thickness decreased linearly with the height of the tube at a rate of 0.05mm/mm. Significant deformation was observed with thin-walled constructs more than 1cm in height. Similar deformation was observed in particle-free constructs around 2cm in height. This effect sets limitations on the design (wall thickness) and dimensions (construct height) of cell-laden constructs in order to maintain structural homogeneity.

Figure 3.5. Fluorescent bead encapsulation within tubular constructs. **(a)** Fluorescent beads were encapsulated homogeneously and with high density within tube walls. **(b)** Customizable cross-section of fluorescent bead-laden tube with high fidelity; 'triangle' cross-section shown. Magnified image in inset shows wall thickness decreasing effect. Wall boundaries indicated by white arrows. **(c)** Thick-walled (top) and thin-walled (bottom) tubular constructs with 'window' (left) and 'triangle' (right) cross-sections. **(d)** Noticeable deformation (wall thinning and structural collapse) toward the tail end of tubular constructs due to light attenuation caused by presence of fluorescent beads.

By comparing the level of deformation observed in fabricated constructs with and without encapsulated beads, it is logical to conclude that the presence of micro-beads had a significant effect on construct deformation. A plausible explanation for the deformation effect could be that light was attenuated towards the tail-end of the construct during fabrication as the opaque particles absorbed more light along the way, causing the BB to gradually weaken as it propagated through the solution. The ability for the weakened BB to crosslink the polymer was reduced, thus leading to the

wall thinning effect. A possible way to improve on the BB printing technique and minimize this limitation is to optimize the “self-healing” property possessed by BBs.

The self-healing mechanism allows the BB to reconstruct itself after being obstructed by an opaque particle (cell in suspension) in its propagation path[131]. As illustrated in Figure 3.6(a), the BB core is able to reform after being obstructed as surviving peripheral rays continue to propagate and undergo constructive interference behind the opaque particle[129]. Figure 3.6(b) demonstrates the capability of the self-healing mechanism in real life experiments. This mechanism enhances the ability of the BB to continue propagation through colloidal suspensions i.e. cellular solutions. It is possible, through optical engineering and laser manipulation, to optimize the effects of this self-healing property and allow maximum BB propagation through cell suspensions, thus reducing the amount of deformation on printed constructs. Although this property was not explored in this dissertation, it would be a logical next step for future work.

Figure 3.6. Self-healing property of BB. (a) Schematic illustration of BB reformation after initial obstruction. (b) Experimental image captured of BB reformation after disruption. (Adopted from ref.[129])

3.3.3 Fabrication of scaffolds with microscale features

Scaffolds with microchannels

TE scaffolds with built in microchannels have been studied extensively for TE applications[237]. The presence of pores or microchannels increases the surface area of the construct and improves nutrient delivery to cells thus keeping the construct viable for longer periods[174]. However, the density of microchannels built into the scaffold affects the mechanical strength of the construct[237], thus a balance between cell survival and structural integrity should be found for any implantable scaffold to ensure the scaffold is functional after implantation. A fabrication technique with the ability to control the size, shape, and organization of individual microchannels effectively would be useful for the fabrication of tissue engineered implantable scaffolds and in vitro tissue models.

Using the BB technique, scaffolds with built-in microchannels and controlled grid spacings of 500 μm (Figure 3.7b-c), and 1 mm (Figure 3.7d) were fabricated, demonstrating the ability to customize microchannel feature size. Printed constructs matched accurately with pre-designed translation profiles shown in Figure 3.7(a). With translational speed of 1mm/s, fabrication time was 2min for constructs in Figure 3.7(b-c), and 50s for construct in Figure 3.7(d). The fabrication time was shortened and the printing mechanism simplified compared to existing scaffold printing techniques due to the elimination of the layer-by-layer processing. However, in exchange for reduced fabrication time and process simplicity, the structural designs suitable for printing are limited due to limitations in the printing technique. Constructs with complex architecture, such as those with curved edges or with bends,

which can be produced through the layer-by-layer approach of commercial laser-printing systems, cannot be reproduced with the BB technique.

Figure 3.7. Fabrication of scaffolds with microscale features. **(a)** View point and stage translation profiles for the fabrication of scaffolds shown in following images. **(b)** PEGDMA scaffold with 500µm grid spacing. **(c)** GelMA scaffold with 500µm grid spacing. **(d)** GelMA scaffold with 1mm grid spacing. **(e-f)** Rhodamine stained GelMA scaffold under higher magnifications showing print resolution around 80µm. Scale bars: (b-d) 1mm (e) 500µm (f) 100µm.

Print resolution

A high print resolution is advantageous for the fabrication of TE constructs as it enables greater precision over cell placement and scaffold architecture, thus enabling more accurate replication of the 3D cellular environment in our native tissue[240]. Extrusion and inkjet-based printing techniques, which are the most commonly used printing techniques in TE, usually have poor resolutions above 100 µm[238], while the resolution of laser-based techniques can be improved to less than 100 µm[239] or even to the nanoscale with two-photon polymerization[240], although the application of the two-photon polymerization technique for TE has been shown to be detrimental

to cell viability due to cytotoxic and phototoxic effects[241,242]. From Rhodamine stained scaffolds fabricated using the BB technique (Figure 3.7e-f), print resolution was found to be around 80 μm , which is an improvement from conventional extrusion and inkjet based techniques, and comparable to laser-based printing techniques. The demonstrated print resolution of 80 μm shown here can be further improved and controlled by manipulating the size of the BB used for printing as described in Chapter 5 of this dissertation where print resolution was shown to be improved to 25 μm .

Synthetic and naturally-derived polymers

Hydrogels are a commonly used material for TE scaffolds due to their biomimetic properties. They consist of either synthetic polymers or natural polymers which have their own unique advantages over the other. For example, synthetic polymers are engineered artificially in vitro and thus have a higher capacity to be chemically modified to achieve the material properties that are required i.e. mechanical strength, chemical composition etc. Natural polymers on the other hand inherently possess higher biocompatibility with cells due to their synthesis in vivo, however they often lack mechanical strength compared to synthetic polymers where mechanical properties can be tailored through chemical modification. Thus, the choice between synthetic polymers or naturally-derived polymers depends on the specific requirements of the user and the objectives of the research study. A fabrication technique with the flexibility to print with multiple hydrogel materials that cater to the specific requirements of the user would provide added convenience and be beneficial for TE applications. Using the BB technique, constructs made of both synthetic polymer (PEGDMA, Figure 3.7b) and naturally-derived polymer (GelMA, Figure 3.7c) were fabricated with 500 μm grid spacing each, demonstrating the versatility of the technique to print with multiple commonly used hydrogel materials which is desirable in the field of TE.

3.4 Summary

In this chapter, the advantages of the BB additive manufacturing technique for the fabrication of hydrogel constructs was demonstrated over conventional techniques and how the technique works is described, including all the equipment and materials required. The unique characteristics of a BB (non-diffractive, high depth-of-field) were utilized to fabricate hydrogel constructs of interest in the field of TE, which has never been reported in the literature. Anatomically relevant, high aspect ratio tubular constructs simulating small diameter vascular grafts - which commonly require the concurrent printing of support structures using conventional techniques - with customizable height, cross-section, and wall thickness were successfully fabricated with significantly reduced fabrication time compared to other hydrogel printing techniques; the fabrication time was effectively reduced by more than half. Encapsulation of fluorescent beads (simulating cells) within the tube walls was also successfully demonstrated with this technique as a proof-of-concept for subsequent chapters where the printing technique will be used for direct cell encapsulation. Finally, 3D hydrogel scaffolds with controlled microscale features and in-built microchannels were fabricated with both naturally-derived and synthetic polymers using the BB technique, showcasing its superior print resolution compared to conventional printing techniques and flexibility. Overall, the technique displayed strong potential to be applied in the field of TE in future.

4. Environmental effects on self-assembled endothelial network formation in hydrogels

4.1 Introduction

In the previous chapter (Chapter 3), a hydrogel printing technique using BBs was studied and displayed significant advantages over conventional printing techniques. No cells were used in any of the experiments and only acellular hydrogel constructs were fabricated. A variety of hydrogels are known to be capable of providing a biomimetic and conducive environment for cell survival and growth. In the field of TE, hydrogels are often the material of choice due to these favourable properties. To meet the objectives of this dissertation, the BB printing technique should demonstrate cytocompatibility and be able to print with live cells, specifically ECs as it is intended to address the challenge of in vitro pre-vascularization. However, before proceeding with cell encapsulation experiments using the BB printing technique (covered in Chapter 5), it is necessary to study how cells behave and function when encapsulated in hydrogel material. To ensure that subsequent cell encapsulation experiments are successful, we need to understand the various properties of the hydrogel ECM that affect cell viability and the ability of encapsulated ECs to self-assemble into endothelial cords (simulating our native individual blood vessels) and networks (simulating our native vascular system).

Most tissues in our bodies are vascularized and can be broken down into 2 distinct components: (1) Cellular and ECM material comprising a multitude of cell types, (2) A vascular network made of ECs responsible for carrying blood nutrient delivery to the entire tissue. In the field of TE, much effort has been made to produce pre-vascularized tissue constructs in vitro which would also consist of these 2 distinct

components. After implantation of a TE construct into a patient, be it pre-vascularized or not, anastomosis with the host vascular system is required for the implant to be fully functional and sustainable in the long run. Successful anastomosis requires adequate vasculogenic and angiogenic processes to occur within the bulk ECM material of the TE construct which is dependent on the ECM properties. Thus, the study of how ECs behave in bulk hydrogel ECM material is also required for the development of functional vascularized tissue constructs in the future.

Collagen type I is a common protein found in many tissues which undergo angiogenesis and known to promote angiogenic sprouting [192]. Cells encapsulated in collagen gels readily degrade, migrate, and proliferate within the gel making it a suitable scaffold for many TE applications [193]. Collagen gels have been reported to promote the organization of bovine adrenal cortex endothelial cells (BACEC) into lumenized tube networks[194]. Human microvascular endothelial cells (HMVEC) have also been shown to form lumenized tubes when co-cultured with fibroblasts in collagen gel[195], and through angiogenic sprouting in microfluidic devices[196]. Mesenchymal cell-dependent branching network formation was also reported for bovine adrenal cortex-derived microvascular endothelial cells (BME) and Bovine aortic endothelial cells (BAEC) encapsulated in collagen gel[197]. Given that HUVECs are often used in vasculogenic and angiogenic models, and vast literature exists of studies done on these cells, an understanding of HUVEC characteristics in collagen gel is imperative.

HUVECs cultured in 3D fibrin gel have been shown to readily form 3D branched networks with clearly defined lumen[94,95,97]. In collagen gel however, the formation of these lumenized networks are not well defined. Studies have found that monolayer cultures of HUVECs supplemented with collagen form branched networks

that mimic non-lumenized premature vasculature[198–200]. The presence of skin fibroblasts were found to provide the necessary signals leading to vessel maturation[198]. It has also been reported that HUVECs encapsulated in 3D collagen gels only assembled into lumenized 3D branched networks[201] with supplementing exogenous factors like PMA[199] or thrombin[200], although some studies did not clearly show the formation of patent lumen to substantiate these claims. For example, the images of “capillary-like structures” found in a previous study[202] were compared to images of “no tube formation”, alluding to the claim that capillary-like tubes were formed spontaneously. However, no images of patent lumen were given as evidence of this claim. Other studies reported that lumenized branching networks formed naturally without the addition of exogenous factors[202–204]. This disparity in the literature is investigated in this chapter.

First, the effects of collagen gel concentration on endothelial sprouting and cell viability was studied. Subsequently, the ability of the cells to form branched networks when fully encapsulated by collagen gel was investigated. The effects of surface treatment of the glass substrates on the formation of branched network was also studied. Confocal imaging was performed on branched networks formed to observe if these endothelial networks were lumenized or remained a flat monolayer.

Previous studies found in literature have shown that HUVECs encapsulated in spatially confined collagen channels formed lumenized tubes along the channel length without the addition of exogenous proteins, and the tubes’ dimensions could be controlled by varying the collagen channel width[36,103,104]. Through the experiments performed in this chapter, this effect was also studied and how spatial confinement of the cells affects the self-assembly of HUVECs into lumenized tubes.

4.2 Methods and materials

4.2.1 Cell culture

All cells were cultured in a standard cell culture incubator (Forma Scientific), in a 5% CO² environment at 37°C. HUVECs (Lonza) were cultured in fully supplemented endothelial cell growth medium (EGM-2 bulletkit; Lonza) with 1% Penicillin/Streptomycin (P/S). P3-5 HUVECs were used for all experiments. Medium was changed every 3 days and cells passaged at 80-90% confluence.

4.2.2 Cell encapsulation in collagen and collagen/fibronectin gel

Collagen gelling procedures were carried out according to supplier instructions. Briefly, the appropriate volumes of 10X PBS, 1 N NaOH solution, DI water, and Collagen I rat tail solution (Corning) were mixed to produce pre-polymer solutions at varying concentrations (Refer to Appendix F for reconstitution protocol provided by manufacturer). For collagen/fibronectin samples, bovine fibronectin (Sigma) was added to the pre-polymer solution at 1.7µg/ml and 3.3µg/ml. The pre-polymer solutions were kept on ice to prevent rapid polymerization. Cells were trypsinized, centrifuged, and resuspended in the aforementioned pre-polymer collagen solutions at varying concentrations. These collagen/cell suspensions were then pipetted onto a coverslip with attached spacer (150µm thickness, 9mm hole diameter, Sigma). Another coverslip was then applied topically to sandwich the cell suspension between the 2 coverslips separated by a distance of 150µm, after which the samples were incubated at 37°C for 30min for full crosslinking. The top coverslips were then removed to expose the top surface of the cell-laden gel. These samples were kept in 6-well plates and maintained in EGM-2 medium for culture. Medium was changed every 2 days.

4.2.3 Cell encapsulation in Fibrin gel

Cells were suspended in 2.5mg/ml bovine fibrinogen solution (Sigma) and pipetted to dissociate cell pellets. 50U/ml bovine thrombin solution (Sigma) was then added to the cell suspension and pipetted continuously for 1 minute until solution viscosity increased for homogeneous cell distribution. The final volume ratio of 50U/ml thrombin solution to 2.5mg/ml fibrinogen solution was 1:100 and final cell concentration was 2×10^6 cells/ml. The viscous solution was pipetted onto the sample platform and incubated at 37°C for an additional 15min for full crosslinking.

4.2.4 Preparation of samples with varying cell density gradients

Different cell encapsulation protocols were used to produce samples with varying cell density gradients within the gel as illustrated in Figure 4.1. Three different samples were produced: **(Sample 1)** Homogeneous cell distribution, **(Sample 2)** More cells at bottom, **(Sample 3)** More cells on top.

To produce homogenous samples (Sample 1), the gels were flipped continuously during the crosslinking process to prevent gravity from pulling cells to one side. For the heterogeneous samples (Sample 2 and 3), no flipping was performed during crosslinking thus allowing the effects of gravity to naturally produce cell density gradients within the sample. The difference between sample 2 and sample 3 is that after full crosslinking, sample 3 was flipped upside down and the top coverslip (red coverslip in Figure 4.1) was removed.

Figure 4.1. Procedure for the preparation of samples with controlled cell density gradients.

4.2.5 Sprout length and area coverage analysis

ImageJ software was used to quantify sprout length and cell area coverage. Sprout lengths were measured manually using a cut-off length of 30 μ m and added up to quantify total sprout length per image. Cell area coverage was quantified using the automated area measurement tool provided by the software which measured the total area of cells per image. The total cell area was presented as a percentage of the total area of an image.

4.2.6 Sigmacote treatment

Sigmacote treatment (Sigma) was carried out according to manufacturer instructions. Briefly, after autoclaving, the glass coverslips were fully immersed in undiluted Sigmacote solution for 5 seconds. The coverslips were left to air dry at room temperature in a laminar flow hood followed by subsequent rinsing with DI water before use.

4.2.7 Cell staining

4.2.7.1 *Live/dead assay*

Cell-laden collagen gels (5mg/ml and 7mg/ml) were stained at day 4 using live/dead assay kit (Invitrogen) according to the manufacturer's instructions. Briefly, Calcein AM (2 μ M) and Ethidium homodimer-1 (4 μ M) were added to sterile PBS and vortexed. Cell-laden gel samples were rinsed twice with PBS before submerging in aforementioned live/dead solution for 30min at 37°C. Live/dead solution was removed and the sample was rinsed twice with PBS before imaging under fluorescent microscopy. Cells were counted using ImageJ to analyze cell viability. Images were captured at six random locations, along multiple z-planes, and the total number of live cells (L) and dead cells (D) were added up to calculate the total cell number (T). Cell viability was calculated as the percentage of L/T.

4.2.7.2 *Immunofluorescence staining*

Cell-laden collagen/fibronectin samples were stained at day 2, 4, and 6. Samples were rinsed with PBS and fixed with 10% formalin solution (Sigma) for 30min. Formalin solution was removed and 0.1% triton X-100 was added to the samples for 30min to permeabilize the cells. Triton X-100 solution was removed and the samples were soaked in 1% BSA (blocking) solution for 2 hours. BSA solution was removed and Anti-CD31 rabbit polyclonal primary antibody (1:100 dilution, Abcam) was added to completely submerge the samples overnight at RT. Primary antibody solution was removed and Goat Anti-rabbit secondary antibody (1:100 dilution, Abcam) was added to completely submerge the samples for 6 hours at RT. Secondary antibody solution was removed and Rhodamine-Phalloidin (1:1000 dilution) was added for 1 hour at RT for actin staining. Excess solution was removed and DAPI (1:1000 dilution) was added for 4.5min for nuclei staining. Excess DAPI solution was removed and the samples were soaked in PBS at 4°C until required for confocal imaging. Samples were rinsed

twice with PBS between each of the steps mentioned in this section to thoroughly remove excess solution.

4.3 Results and Discussion

4.3.1 Effects of collagen concentration

Collagen solutions were prepared with varying final concentrations of 3, 5, and 7mg/ml for cell encapsulation. Cells were suspended in the collagen solutions at a concentration of 1.6×10^6 cells/ml for all samples before crosslinking. After 2 days in culture, images were captured of cells encapsulated within 3mg/ml (Figure 4.2a), 5mg/ml (Figure 4.2b), and 7mg/ml (Figure 4.2c) collagen gel and the amount of endothelial sprouting, if any, was quantified. Results showed that as collagen concentration increased, there was a significant decrease in total sprout length per image (Figure 4.2d). Endothelial sprouts are indicated by black arrows in Figure 4.2(a-c). This result can be attributed to the changing mechanical properties of gels with different concentrations causing variations in sprout length[205,206]. Live/dead staining was performed at day 4 to quantify the effects of varying collagen concentration on cell viability. After staining, fluorescent images were captured of cells encapsulated within 5mg/ml (Figure 4.2e-g) and 7mg/ml (Figure 4.2h-j) collagen gel with green representing live cells and red representing dead cells. Live/dead quantification revealed that an increase in collagen concentration from 5mg/ml to 7mg/ml significantly reduced cell viability from 88% to 59% (Figure 4.2k). This result has been previously reported in the literature, suggesting that the more densely packed crosslinking structure of the higher concentration gels reduced permeability for nutrient diffusion thus resulting in decreased cell viability[207,208].

Figure 4.2. Effects of collagen concentration on endothelial sprouting and viability. Cell concentration 1.6mil/ml. Brightfield images of day 2 HUVEC cultures in collagen concentrations (a) 3mg/ml, (b) 5mg/ml, and (c) 7mg/ml. Black arrows indicate elongated cell sprouts. (d) Total sprout area per image decreased with increasing collagen concentration (n=5). (e-g) Merged images of live/dead stained day 4 cells cultured in 5mg/ml and (h-j) 7mg/ml collagen. Green represents live cells while red represents dead cells. (k) Cell viability decreased with increasing collagen concentration (n=6). Scale bars are 100μm. Error bars represent SEM (*p<0.05).

4.3.2 Effects of cell distribution within gel

Cell-laden gels were prepared with varying cell distribution within the samples as described in section 4.2.4. At day 2, cells were imaged at 3 planes along the thickness of the sample and each plane separated by a distance of 40 μm in the z-direction as

illustrated schematically in Figure 4.3(m). Cells in sample 1 (Figure 4.3a-c) and sample 3 (Figure 4.3j-l) remained rounded and showed no signs of network formation at all planes. Cells in sample 2 were observed to attach and sprout to form branched networks only at plane 1 as shown in Figure 4.3(d,g) whereas cells at higher planes remained rounded as shown in brightfield and fluorescent images in Figure 4.3(e,f,h,i). Cells consistently possessed rounded morphology in samples 1 and 3 throughout the sample thickness. These results indicate that at the gel-coverslip interface, cell density must be sufficient in order for branched networks to form. Regardless of cell density, cells fail to form branched networks at planes above the gel/coverslip interface, indicating that the branched networks formed are confined to a 2D plane and attachment onto the coverslip is necessary for network formation.

Figure 4.3. Cell morphology of day 2 cells at different planes (40μm apart) cell-laden collagen gel. All samples were cultured with a cell density of 5mil/ml on autoclaved coverslips. **(m)** Schematic diagram showing the relative z-positions of images captured. 3 planes (plane 1, plane 2, and plane 3) were imaged with plane 1 at the gel-coverslip interface and each subsequent plane separated by a distance of 40μm. Brightfield images of cells are shown at **(a,d,j)** plane 1, **(b,e,k)** plane 2, **(c,f,l)** plane 3. **(a-c)** Sample 1; homogenous cell density. Cells at all planes remained rounded and failed to attach onto the coverslip. No branched networks were observed throughout the sample. **(d-f)** Brightfield and **(g-i)** fluorescent images of Sample 2; higher concentration of cells towards the bottom of the sample. Cells in plane 1 (red border) demonstrated noticeable sprouting and branched network formation while cells on higher planes remained rounded. Dark cell patterns captured in **(f)** are the same cells shown in **(d)** but out of focus. **(j-l)** Sample 3; higher concentration of cells towards the top of the sample. Cells in all planes remained rounded, no branched network formation. Scale bars represent 100μm.

4.3.3 Effects of substrate treatment

After observing how branched networks form only at the gel-coverslip interface, it was tested to see if treatment of the coverslip surface would affect branched network morphology to further support the results. Coverslips were treated by autoclaving or Sigmacote solution (hydrophobic). HUVECs were then encapsulated within 3mg/ml collagen gel at concentrations of 3, 5, and 10×10^6 cells/ml on top of autoclaved coverslips (Figure 4.4a-c) and on Sigmacote treated coverslips (Figure 4.4d-f). The cell area coverage was quantified per image and showed that cells cultured on Sigmacote treated coverslips (an anti-adherent) had significantly reduced area coverage compared to cells cultured on autoclaved coverslips (Figure 4.4g). Cells on all samples were observed to form branched networks, however the sprouting branches on autoclaved coverslips were thicker (30 μm) compared to the sprouting branches on Sigmacote treated coverslips (12 μm), which led to the difference in cell area coverage quantified. All cells located away from the gel-coverslip interface remained rounded.

Figure 4.4. Effects of substrate treatment on cell morphology. **(a-c)** Brightfield images of day 2 cells cultured on autoclaved coverslips. **(d-f)** Brightfield images of day 2 cells cultured on coverslips which were autoclaved followed by anti-adhesive sigmacote treatment. **(g)** Cell area coverage (%) was quantified per image. At all cell concentrations (3, 5, 10mil/ml) there was a significant reduction in cell area coverage after sigmacote treatment. Scale bars are 100 μ m. Error bars represent SEM (n=5, *p<0.05).

There were significant differences in cell morphology at day 2 and day 4. For autoclaved (Figure 4.5b,e) and Sigmacote treated coverslips (Figure 4.5c,f), the branched networks formed at day 2 regressed into clumps of individual rounded cells at day 4 (indicated by black arrows), possibly due to increased hydrophobicity caused by the autoclaving and Sigmacote treatment. This observation is consistent with reported literature where cells cultured on hydrophobic substrates (PDMS) managed to attach initially, but eventually detach after long term culture[209]. In contrast, cells on untreated coverslips maintained their branched networks after day 4. Cell area per image was quantified to show that while the cell area for untreated coverslips

increased from day 2 to day 4, cell area for the autoclaved and Sigmacote treated coverslips decreased due to branched network regression (Figure 4.5g).

Figure 4.5. Cell morphology at 2 and 4 days with different coverslip treatment. **(a-c)** Cells at day 2 were observed to sprout and attach onto the coverslip for all samples. **(e-f)** By day 4, cells cultured on autoclaved and sigmacote treated coverslips seemed to detach from the coverslip and become rounded, indicated by black arrows. **(d)** Cells cultured on untreated coverslips remained attached on coverslip. **(g)** Cell area coverage decreased from day 2 to 4 for autoclaved and sigmacote treated coverslips but increased for untreated coverslips. Scale bars are 100 μ m. Error bars represent SEM (n=3, *p<0.05).

4.3.4 Lumenization and effects of ECM geometry

Branched network formation was consistently observed in these experiments, although confined to a 2D plane at the gel-coverslip interface. To determine whether these branched networks consisted of lumenized tubes, confocal imaging was performed on DAPI/actin stained networks. Figure 4.6(a-c) depicts an area of the sample which contains branched networks formed by HUVECs. Each image has been sectioned at different locations (sectioning locations denoted by the green and red

lines), and the cross-sectional images are found adjacent to the planar image. From the cross-sectional images, a flat cell monolayer was observed (indicated by teal arrows) with no signs of lumenization.

Reports found in the literature have demonstrated non-lumenized network formation in collagen gels[198–200], but contradicts other studies that report lumenized tube formation of HUVECs encapsulated in collagen ECM[202–204]. Several experimental differences can be found from comparing these past reports which could have caused the variation in results including the cell encapsulation procedure and the medium supplements used. In particular, the collagen used in studies that reported non-lumenized network formation were supplied commercially (BD Biosciences)[197][199], while studies that reported lumenized tube formation[202,203] used manually harvested collagen from rat tail tendons following a common protocol[214], resulting in a difference in collagen gel composition which could have caused the variation in results. Further experiments should be conducted to investigate this observation. These past studies used histological staining combined with brightfield and electron microscopy to obtain images which were later analyzed to observe lumenogenesis. In the current study, the use of fluorescent staining techniques and state-of-the-art confocal microscopy to observe lumenogenesis provides compelling evidence to indicate that branched networks formed by HUVECs in 3D collagen gels, without the addition of exogenous proteins for enhanced performance, do not consist of lumenized tubes.

Figure 4.6. Lumenization of branching networks. **(a-c)** Confocal images captured of networks formed at day 2 showed that they maintained a flat morphology and no lumen formation was observed as indicated in the cross-sectional views (teal arrows). Cell concentration 10mil/ml. Scale bars represent 50 μ m.

In some of the samples, portions of the cell-laden collagen gel were dislodged from the coverslip and were folded, thus creating areas of spatial confinement for the encapsulated cells as illustrated in the schematic diagram in Figure 4.7. Fluorescent images of DAPI/actin stained HUVECs were captured at areas where the gel was folded, and also at areas where the gel remained flat and attached to the coverslip to compare cell morphology. HUVECs encapsulated within the flat portion of the gel were observed to possess the same morphological characteristics as those observed in Section 4.3.2, where cells at the gel-coverslip interface maintained flat morphology (Figure 4.7a) while cells fully encapsulated within the collagen ECM at higher planes remained rounded (Figure 4.7b). In contrast, at the folded portions of the gel within the same sample, cells appeared to form aligned endothelial cords as shown in Figure 4.7(c-f). Z-position between images was varied to bring different cords into focus as indicated by the white arrows in the figure. The formation of endothelial cords at folded portions of the gel was consistent throughout the study.

From reviewing the literature, it was found that fibrin gels and collagen gels were commonly utilized in different ways for in vitro vascularization. Fibrin gel is commonly used in current literature for the formation of perfusable vascular networks in vitro through the encapsulation of HUVECs within bulk fibrin gel (not spatially confined)

and left to self-assemble into lumenized endothelial networks which have been shown to be perusable[94-95]. Collagen, on the other hand, is commonly used for the encapsulation of ECs within spatially defined collagen channels[103-104], which induce self-assembly of the ECs into endothelial cords proportional to the channel size. This difference can be explained from the current results where it was determined that while HUVECs are able to self-assemble into endothelial cords consistently within spatially confined collagen channels, the same cord formation is not observed in HUVECs encapsulated in bulk collagen gel, indicating that encapsulated HUVECs responded to spatial cues which induced them to form cords and that spatial confinement within collagen channels is necessary for lumenization. This result has not been reported in the literature and further experiments should be conducted to explain the mechanisms behind this result.

Figure 4.7. Effects of ECM geometry on lumen formation. Schematic diagram illustrates folding of cell-laden gel samples which created areas of spatial confinement. **(a-f)** Fluorescent images of DAPI/actin stained cells cultured within the same sample. Cell concentration 1.1mil/ml. **(a-b)** When the collagen

ECM remained flat and firmly attached onto the coverslip, no lumen formation was observed **(a)** and cells at higher planes remained rounded **(b)**. Within the same sample, lumen formation was observed when the collagen gel no longer attached onto the coverslip and did not maintain a flat shape but instead was folded **(c-f)**. EC lined walls indicated by white arrows. Scale bars represent 100µm.

4.3.5 Effect of collagen and fibronectin concentrations on HUVEC morphology and phenotype

Collagen and fibronectin are common components of ECM which support cell survival.

The addition of fibronectin to collagen blends is known to promote EC adhesion and tube formation[46,102,210], and is commonly used in vascularization research[93,211–213]. In this study, HUVECs were encapsulated within 3D hydrogel constructs of varying collagen and fibronectin concentrations to study its effect on HUVEC morphology and phenotype.

Figure 4.8. Effects of collagen/fibronectin concentrations on HUVEC morphology and phenotype. Blue represents DAPI, red represent actin, green represents CD31 staining. Rounded cell morphology was found in all samples; no sprouting and network formation observed. Cell seeding density 5×10^6 cells/ml.

Figure 4.8 shows confocal images of cells stained with DAPI (nuclei; blue), Rhodamine Phalloidin (actin; red), and CD31 (endothelial marker; green). The results showed that, similar to the previous experiments in pure collagen, cells which had no contact with

the substrate surface remained rounded with no cell-cell interconnection and no vascular networks were formed regardless of collagen/fibronectin concentration. As days increased, cell spreading indicated by actin staining was observed to reduce in all cases indicating failure to attach onto the ECM. Despite the failure to attach, cells in pure collagen (top row) demonstrated increased expression of CD31 with time indicating an increase in endothelial phenotype. High fibronectin concentration coupled with low collagen concentration (bottom row) resulted in decreased cell spreading (hardly any actin expression) at all time points as compared to pure collagen samples even though endothelial marker was still expressed. Overall, the addition of fibronectin to collagen blends did not induce HUVEC attachment and network formation compared to pure collagen.

4.3.6 Cell morphology within 3D fibrin gel

Figure 4.9. HUVECs encapsulated within fibrin gel at 2×10^6 cells/ml. **(a-c)** Cells imaged at various planes within the gel samples demonstrated consistent elongated and vessel like morphology compared to the rounded morphology in collagen gel. **(a-c)** HUVECs imaged at various planes within the gel sample showing sprouting. Scale bars are 100 μ m.

From the current experiments with collagen gel, vascular network formation at planes above the substrate was not observed as cells failed to sprout when fully encapsulated in collagen ECM. In contrast, HUVECs encapsulated in fibrin gels readily formed 3D networks which were observed to sprout in all directions including the z-

direction. At higher planes above the substrate, cells fully encapsulated in fibrin ECM displayed elongated vessel-like morphology unlike the rounded morphology displayed in collagen. Images captured in Figure 4.9(a-c) were captured 40 μ m apart in the z-direction and features branching patterns and elongated cell morphology.

From this result, fibrin gel demonstrated greater vascularization potential compared to collagen gel when HUVECs are encapsulated within bulk hydrogel (without spatial confinement). Thus, in order to ensure timely and effective infiltration and anastomosis of the host vascular system with a TE construct post implantation, the use of fibrin gel as the bulk ECM material for the implant is recommended over the use of collagen gel. This result is aligned with previous studies where it was found that fibrin but not type I collagen induced angiogenesis in vivo[215]. Studies have shown that this difference could be attributed to varying integrin expressions in ECM with different compositions. Expression of alpha v beta 3 integrin, the receptor for fibrin gel matrix, is responsible for angiogenic regulation and response to growth factors such as VEGF, and has been shown to be differentially regulated by varying ECM[216]. It has also been shown that fibrin proteins significantly upregulated the expression of alpha v beta 3 integrin compared to collagen gel in Human Dermal Microvascular Endothelial Cells (HDMEC)[217], and the differential regulation of angiogenic sprouting was observed using 3D in vitro angiogenic models comparing fibrin and type I collagen[218]. These studies indicate that both the mechanical properties and chemical compositions of ECM play a significant role in angiogenic regulation.

4.4 Summary

In this chapter, it was found that although it is common in the literature to refer to in vitro vascular networks as “3D”, “capillary-like”, “tubular”, and “lumenized”, the

actual state of the networks may not be as described. Evidence was presented to show that HUVEC networks cultured in 3D collagen gels - which look similar to studies found in the literature under microscopy - were confined to a 2D layer and were non-lumenized. As revealed in Figure 4.3, the cells remained rounded when fully encapsulated in collagen ECM and failed to form 3D networks effectively when compared to fibrin gel. This find could possibly lead to more prominent use of fibrin as bulk ECM material compared to collagen in future vascularization studies.

It was demonstrated that collagen gel concentration significantly affected cell viability and extent of sprouting. It was also found that cells failed to form branched networks at higher planes above the gel-coverslip interface, indicating that cell attachment onto the glass coverslip was necessary for network formation and these networks are confined to a 2D plane. This is further emphasized by the result that substrate treatment had a significant effect on network morphology, with the thickness of individual branches decreasing and increased cell detachment due to increased substrate hydrophobicity. The majority of cells that successfully attached onto hydrophobic substrates early on were observed to detach to form cell clusters with rounded morphology by day 4. It is thus logical to conclude that there are interactions between the cells and the substrate which contributed to this result, although further work should be carried out to substantiate this claim i.e. focal adhesion protein staining.

Through confocal microscopy it was found that the networks formed at the gel-coverslip interface were non-lumenized. However, the formation of endothelial cords was observed at spatially confined regions of collagen. When encapsulated in bulk fibrin gel without spatial confinement, HUVECs readily formed branching networks which were found at all planes throughout the gel thickness, indicating that ECM

composition significantly affects cell morphology and that fibrin gel offers a more conducive environment for self-assembled vascular network formation.

From the results in this chapter, we can conclude that at the right concentrations, collagen gels do provide the necessary conditions for cell survival and endothelial sprouting. However, the endothelial sprouts are non-lumenized and do not replicate the architecture of our native blood vessels. In order for HUVECs to self-assemble into endothelial cords in collagen gels, the cells need to be encapsulated within spatially confined hydrogel ECM.

5. Cell-laden fiber fabrication for endothelial cord formation using Bessel Beams

Work presented in this chapter is largely based on the following publication: **Liew, Wen Loong Andy, and Yilei Zhang. "Cell-laden gelatin methacryloyl fibres fabricated using bessel beams for controlled endothelial cord formation." *Biomedical Physics & Engineering Express* (2018) 4 045009.**

5.1 Introduction

As previously discussed in Section 2.1.2 (Chapter 2), there is a need for in vitro pre-vascularization techniques capable of producing pre-vascularized TE constructs prior to implantation in the field of TE. The pre-vascularization of TE constructs minimizes the time required for anastomosis with the host vascular system after implantation to ensure long term sustainability and functionality of the implant[36]. It also brings us a step closer to fabricating complex, vascularized, biomimetic tissues and organs in vitro.

In Chapter 3, a laser-based printing technique capable of fabricating acellular 3D hydrogel constructs using BBs was introduced. In Chapter 4, cell behaviour when encapsulated in hydrogels was studied and the factors that affect cell viability and endothelial network formation were determined. In this chapter, the same printing technique from Chapter 3 is combined with the knowledge gained in Chapter 4 to address the need for in vitro vascularization techniques by applying the printing technique for the direct encapsulation of live HUVECs to produce endothelial cords in vitro.

Although collagen gel promotes endothelial sprouting, as seen in Chapter 4, the sprouts are non-lumenized when encapsulated in bulk gel (Figure 4.6, Chapter 4).

However, when encapsulated in spatially confined gel, HUVECs were shown to self-assemble into endothelial cords (Figure 4.7, Chapter 4). In light of these results, a method to use the BB printing technique to encapsulate HUVECs within GelMA fibers was developed to provide the spatial confinement necessary for self-assembled cord formation.

First acellular fibers were printed using the BB technique and showed its unique advantages compared to conventional fiber fabrication techniques. Based on mathematical models, the effects of varying the laser set up on the BB core diameter were studied, which in turn controlled the diameters of the printed fibers. Live cells were then incorporated into the printing process to produce cell-laden GelMA fibers. The cytocompatibility of the printing technique was assessed through live/dead staining and cell proliferation quantification and compared to conventional bioprinting techniques. Finally, confocal imaging was performed on cell-laden fibers at specific time points post fabrication to determine the formation of self-assembled endothelial cords.

This chapter presents the first report of BBs being used for the encapsulation of live cells in hydrogel fibers with TE applications.

5.2 Methods and materials

5.2.1 GelMA synthesis

10%w/v Gelatin type A from porcine skin was dissolved completely in Dulbecco's Phosphate Buffered Saline (DPBS) at 60°C for 1 hour using a magnetic stirrer at 600 rpm. 0.2% (low), 1.4% (medium), and 15% (high) v/v Methacrylic Anhydride (MA, sigma) was added to the solution in aliquots and kept at 50°C for 2 hours. The volumes of MA added were based on previously published literature using similar concentrations[109,181,182]. A 5X dilution of the GelMA solution was carried out

with Phosphate Buffered Saline PBS (Pre-warmed to 40°C) to quench the reaction. The solution was dialyzed in De-ionized (DI) water for 7 days at 40°C, followed by lyophilization at -90°C for 4 days to obtain a white foamy material which was stored at -80°C until further use.

5.2.2 Optical setup

Figure 5.1. Optical setup for GelMA photopolymerization using BBs. Solid blue lines represent the laser beam; Shaded areas represent Bessel region; Dotted red lines represent the BB core; Solid black arrows indicate laser propagation direction.

The optical setup is shown in Figure 5.1. First, a UV laser (355nm) emits a Gaussian laser beam (solid blue lines) which propagates through lens c₁, mirror m₁, and lens c₂ to produce a collimated beam. It then passes through an aperture which is later used to block out unnecessary peripheral rays during photopolymerization. After passing

through an Axicon ($\alpha=179^\circ$, $n_a=1.5$, Thorlabs), a Bessel region (shaded area) was produced where the high intensity non-diffracting BB core exists (dotted red lines). The laser beam then propagated through a series of mirrors and lenses (m_2 - f_1 - m_3 - f_2 - m_4) which were used to change the laser propagation direction due to space constraint and refocus the laser beam, respectively. After reflecting off mirror m_4 , the Bessel region re-emerges and the BB core produced here was used for photopolymerization of GelMA fibers as it propagated through the pre-polymer solution which was translated using a 3D motion controlled stage (Shanghai Lianyi).

Theoretically, by changing the configuration of optical elements (varying the focal lengths of lens f_1 and f_2), the diameter of the BB core can be tuned, thus the dimensions of fibers polymerized by the BB core can also be controlled (diameter and length). In this study, three different optical configurations were used by varying both f_1 and f_2 (Table 5.1), and each configuration possessed a different Magnification (M) value, which is the ratio f_2/f_1 .

Table 5.1. Focal lengths of lenses f_1 and f_2 used for the three optical configurations to produce fibers of controlled diameter. Magnification (M) is the ratio f_2/f_1 .

Configurations			
	1	2	3
M	0.5	1	1.5
f_1 (mm)	1000	N.A	500
f_2 (mm)	500	N.A	750

5.2.3 Pre-polymer solution preparation

Lyophilized medium-MA GelMA was added to DPBS at 5%w/v and left to dissolve completely at 60°C for 3 hours. Photoinitiator 2-hydroxy-1(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959, CIBA Chemicals) at 0.5%w/v was then added and dissolved into the solution at 60°C for 1 hour to obtain a clear solution. This solution was used for acellular fiber fabrication. For cell encapsulation experiments, additional

steps were performed. HUVECs were trypsinized, counted, and resuspended in GelMA/photoinitiator solutions at 2×10^6 cells/ml to obtain homogenous cell/hydrogel solutions. These cell-laden solutions were used for all subsequent cell encapsulation experiments.

5.2.4 GelMA mechanical testing

For compression tests, acellular GelMA pre-polymer solution was prepared at varying concentrations of 5%, 10%, and 15%(w/v) in DI water at all levels of MA concentration – 0.2% (low), 1.4% (medium), and 15% (high) – total of 9 experimental runs (Table 5.2). Compression tests were performed at room temperature on GelMA samples according to previously published procedures with slight variations[86,109,159]; procedures are identical to PEGDMA mechanical testing in Section 3.2.3. Cylindrical samples were prepared by pipetting 80mL of solution into an 8mm diameter mold and exposing the solutions to UV light (UVF400/600 UV Flood System, 250-650 nm, 50% power setting, 120 s) for crosslinking. The resultant gel samples were 1.6mm in height and retained their 8 mm diameter and cylindrical shape after removal from the mold. 3 replicate samples were prepared for each experimental run. After UV exposure, the samples were removed from the molds and soaked overnight in DI water to ensure complete swelling before mechanical testing. Samples were blotted lightly with Kimtech wipes to remove excess water before being loaded onto an Instron 5566 mechanical tester for compression loading using a 100N load cell. Samples were compressed at a rate of 20% strain/min. Compressive modulus was determined based on the slope of the stress-strain curve.

Table 5.2. Reference table for samples used in mechanical testing. Three levels of GelMA concentration (5%, 10%, 15%) and three levels of methacrylation (0.2% low, 1.4% medium, 15% high) were tested for a total of 9 samples.

Methacrylation level	GelMA Concentration (w/v)		
	5%	10%	15%
Low MA (0.2%)	Sample 1	Sample 4	Sample 7
Medium MA (1.4%)	Sample 2	Sample 5	Sample 8
High MA (15%)	Sample 3	Sample 6	Sample 9

5.2.5 Cell culture

All cells were cultured in a standard cell culture incubator (Forma Scientific), in 5% CO₂ environment at 37°C. For cell expansion, HUVECs were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin (P/S) and were passaged twice per week. After sufficient cell numbers were reached, HUVECs were trypsinized and used for encapsulation experiments; procedures for cell encapsulation are detailed in subsequent sections 5.2.8 and 5.2.9. After cell encapsulation, the cell-laden GelMA fibers were transferred to a 6-well plate and maintained in supplemented DMEM for live/dead assay, or fully supplemented endothelial cell growth medium (EGM-2 bulletkit; Lonza) for cord-formation study.

5.2.6 Acellular fiber fabrication

The fabrication procedure is schematically shown in Figure 5.2. All procedures were carried out at room temperature unless otherwise stated. Acellular pre-polymer solution was added to a 96-well plate and an untreated glass cover slip was used to cover the 96-well such that the surface of the acellular pre-polymer solution was in contact with the square cover slip. The plate was then placed onto the build platform of a translating stage where it was centered and aligned manually. The pre-polymer solution was subjected to multiple UV exposures from the laser in the form of a BB.

Each exposure resulted in a single acellular fiber being produced due to photo-polymerization of the pre-polymer solution. After each exposure, the 3D stage was translated by a distance of 500 μm and the exposure was repeated until sufficient fibers were produced.

Figure 5.2. Schematic diagram of procedure used for acellular fiber fabrication and cell-laden fibers for cord-formation study.

Different M values were used to fabricate fibers of varying diameters ($M=0.5$, 1 , and 1.5). Exposure time was set at 5s for all samples. The exposure time was selected to suit the power of the laser system, which was set at a range of 20-30% power output. After laser exposure, the fabricated fibers attached at one end onto the untreated glass cover slip while the rest of the fiber body remained unattached to any surface. The 96-well plate was then placed into a 37°C incubator for 1 minute to reduce the viscosity of the surrounding un-polymerized solution, after which the coverslip, along with attached fibers, was lifted off the 96-well plate and gently dipped in PBS to remove excess un-crosslinked GelMA solution. After which, the samples were observed under brightfield microscopy.

Based on the laser powers used (50 μ W for M=0.5, 120 μ W for M=1, and 160 μ W for M=1.5) and the exposure time of 5 seconds, the UV dose for each experimental run was calculated; UV dose is known to affect the degree of crosslinking. The calculations of UV dose for all configurations used in the current experiments are provided in Appendix A. The UV dose used in all experiments were higher than the level required for full crosslinking (4860mJ/cm²) based on a study of the relationship between UV dose and the compressive modulus (degree of crosslinking) of GelMA [180]. Furthermore, the UV light used in the cited paper was 365nm which is higher than that used in this chapter which was 355nm. Based on the absorption spectra of Irgacure2959 photoinitiator (Appendix B), 355nm light should cause a higher degree of crosslinking compared to 365nm light. These factors indicate that full crosslinking of the GelMA fibers was achieved in the current experiments.

5.2.7 BB core diameter calculation

BB core diameters could be calculated theoretically using the following equations[145]:

$$\theta \approx \left(\frac{\pi}{2} - \frac{\alpha}{2} \right) \left(\frac{n_a}{n_m} - 1 \right), \quad (1)$$

where θ is the angle between the propagation axis and the plane wave vectors (Figure 5.1), n_a is the refractive index of the Axicon, n_m is the refractive index of the surrounding medium (air), and α is the apex angle of the Axicon.

$$r_b = \frac{2.4048}{k \sin \theta}, \quad (2)$$

where r_b is the radius of the BB core, i.e. the distance from the beam center to the first dark ring when no additional lenses are used behind the Axicon, the constant

2.4048 is the first root of the zeroth order bessel function, and k is the wavenumber of the beam propagating through the medium.

$$M = \frac{f_2}{f_1}, \quad (3)$$

where M is the magnification caused by lenses L_1 and L_2 with focal lengths f_1 and f_2 respectively.

$$r'_b = Mr_b, \quad (4)$$

where the calculated value of r'_b is the resultant BB core radius when a combination of the two lenses L_1 and L_2 are placed behind the Axicon. By combining these equations, the resultant BB core radius (r'_b) could be calculated using:

$$r'_b = M \frac{2.4048}{k \sin \theta} \quad (5)$$

Theoretical calculation details are provided in Appendix C.

5.2.8 Cell encapsulation procedure for live/dead assay

This procedure is similar to the procedure used to fabricate acellular fibers with the exception that the pre-polymer solution was loaded with HUVECs, and the glass cover slip was not used as illustrated in Figure 5.3. After multiple laser exposures, the cell-laden fibers fabricated were unattached to any surface. The laser parameters used were: $M=1$, laser power = 50 μ W, exposure time = 5s. The well was then placed in a 37°C incubator for 1 min to reduce the viscosity of the surrounding unpolymerized GelMA solution causing the polymerized cell-laden fibers to settle to the bottom of the well. The contents of the well were then pipetted and passed through a filter (Corning cell strainer, 40 μ m) to separate the cell-laden fibers from the excess unpolymerized solution. The cell-laden fibers were transferred into a fresh 6-well

plate containing supplemented DMEM to provide nutrients for cell survival and incubated for 3 days.

Figure 5.3. Schematic diagram of procedure used to fabricate cell-laden fibers for live/dead assay.

After 3 days in culture, the cells were stained using the live/dead assay kit (Invitrogen) according to the manufacturer's instructions (refer to Section 4.2.7.1 for detailed procedures). Immediately after staining, the cells were imaged under fluorescent microscopy. Cells were counted using ImageJ software (NIH) to analyze cell viability. Images were captured at five different locations, along multiple z-planes, and the total number of live cells (L) and dead cells (D) were added up to calculate the total cell number (T). Cell viability was calculated as the percentage of L/T.

5.2.9 Cell encapsulation for cord-formation study

The fabrication of cell-laden GelMA fibers for cord-formation study follows the same procedure used to fabricate acellular fibers illustrated in Figure 5.2, except that the pre-polymer solution used was loaded with HUVECs. Following this procedure, cell-laden GelMA fibers attached onto a cover slip were obtained. The entire coverslip,

along with attached cell-laden fibers, was then placed inside a fresh 6-well plate and 1.5ml of fully supplemented EGM-2 medium was added into the well such that the cover slip and attached cell-laden fibers were fully submerged. The 6-well plate was then placed in an incubator until required for staining and imaging. Fully supplemented EGM-2 medium was used in this experiment as it contains growth factors such as Vascular Endothelial Growth Factor (VEGF) which is known to induce tubulogenesis. Medium was replenished at day 2 and 4.

5.2.10 Fluorescent staining

For cord-formation study, fluorescent staining (DAPI and Actin) was performed to visualize the morphology and proliferation of cells encapsulated within GelMA fibers at different time points. Medium was first pipetted out of the well, followed by washing twice with PBS. A volume of 1.5ml Glutaraldehyde (3%) was then added to the well to fix cells for 30min, followed by washing twice with PBS. Subsequently, 1.5ml of 0.1% Triton X-100 was added to permeabilize cells for 30min, followed by washing twice with PBS. Rhodamine-phalloidin (life technologies) was diluted at 1:1000 in PBS and 1.5ml of the diluted solution was pipetted into the well for 1h at room temperature for actin staining, followed by washing twice with PBS. Then 1.5ml of 1:1000 diluted DAPI solution was placed into the well for 4.5 min to stain for cell nuclei, followed by washing twice with PBS to completely remove any residue. After that, 1.5ml of PBS was added to the well to prevent the GelMA from drying up during storage. The 6-well plate was covered in aluminium foil and stored in a 4°C fridge until it was taken out for imaging. This staining procedure was carried out at day 1, 3, and 5. After all samples were stained, Z-stacked fluorescent images were captured using a Zeiss LSM780 confocal microscope and subsequently rendered into 3D views using ImageJ software. Distance between each stack was 1µm. To quantify cell proliferation,

images of DAPI stained nuclei were converted to black and white masks for cell counting using ImageJ software. The cell count for each image was then divided by the fiber area in which the cells were encapsulated and plotted in a graph to observe the change in cell density with time.

5.3 Results and Discussion

5.3.1 GelMA selection

In Chapter 4, we found that fibrin possessed greater vasculogenic potential compared to collagen gels, thus for in vitro vascularization applications, fibrin gel is preferred. Fibrin is a biodegradable protein produced naturally in our bodies as the end product of the blood-clotting cascade that helps to prevent excessive bleeding at injury sites[243]. It is a naturally forming polymer with demonstrated applications in TE due to its low toxicity, low immunogenicity, and ability to encapsulate cells[244]. However, despite its favourable properties, the crosslinking mechanism of fibrin gel relies on the cleavage of fibrinopeptides by thrombin to produce fibrin monomers which then polymerize into insoluble fibrin gel[245]. This makes it incompatible with the BB printing technique studied in Chapter 3 which relies on photopolymerization of the uncrosslinked solution when exposed to UV light.

The successful printing of both synthetic (PEGDMA) and naturally-derived (GelMA) hydrogel was demonstrated in Chapter 3. For direct cell encapsulation, biocompatibility of the ECM material is crucial for cell survival, thus the use of naturally-derived hydrogels are preferred. Gelatin Methacryloyl (GelMA) consists of functionalized gelatin with both methacrylate and methacrylamide groups present allowing it to be photopolymerized and selectively patterned under UV light into crosslinked hydrogel material[176–178]. GelMA also possesses excellent

biocompatibility and supports cell adhesion and viability as demonstrated in numerous reports in the literature[109,179-180]. In Chapter 4, we found that collagen gel does promote cell viability and endothelial sprouting at the appropriate concentrations. As gelatin is derived from the irreversible denaturing of collagen, both gelatin and collagen share similar chemical compositions and amino acid content, thus making GelMA a suitable material choice for cell encapsulation experiments in this chapter.

5.3.2 GelMA mechanical characterization

ECM mechanical properties are known to affect cell behavior[183] such as differentiation[184,185] and proliferation[186]. The different tissues in our bodies possess wide-ranging mechanical properties which are suited for their unique functions. Load bearing tissue such as bone and cartilage require higher strength and stiffness compared to soft tissue like skin and muscle. Thus, it is advantageous for biomaterials applied in the field of TE to have tunable mechanical properties to replicate the stiffness of different tissues. Figure 5.4(a) shows the stress-strain curve obtained from uniaxial compressive tests of GelMA samples with varying concentrations and degrees of methacrylation (MA). Figure 5.4(b) shows the compressive modulus of each of the GelMA samples which followed a distinct trend. Samples at 5% concentration had lower crosslinking densities and were exceedingly soft and difficult to handle during testing compared to higher concentrations, which could have led to variations in the compression test results and contributed to the statistically insignificant results. Compressive modulus was observed to significantly increase as MA level increased for 10% and 15% gels. Similarly, compressive modulus increased significantly as gel concentration increased for all levels of MA. These results demonstrate that the GelMA used in this dissertation possesses tunable

mechanical properties based on hydrogel concentration and degree of methacrylation (MA).

The compressive modulus of GelMA is lower compared to PEGDMA samples in Section 3.3.1, indicating that it has weaker mechanical properties. This makes the use of GelMA unsuitable for the replacement of certain load-bearing tissue that requires higher structural strength. However, for the objectives of this chapter, which is to produce endothelial cords in vitro (soft tissue), high structural strength is not required. A more important property to possess is superior cytocompatibility. As GelMA is a naturally-derived polymer, it inherently possesses superior cytocompatibility compared to synthetic polymers like PEGDMA thus providing a more hospitable environment for the survival of encapsulated HUVECs.

Figure 5.4. GelMA mechanical characterization. **(a)** Compressive stress/strain curve for low-high MA GelMA and varying %(w/v) concentrations. **(b)** Compressive modulus vs GelMA concentration graph revealing significant effects of GelMA concentration and degree of methacrylation on hydrogel stiffness. Error bars represent SEM (* $p < 0.05$, $n = 3$).

5.3.3 Acellular fiber fabrication with controlled fiber diameter

Unlike conventional focused beams, which are focused by propagation of a Gaussian beam through a converging lens into a single high intensity voxel, a BB is created by propagation through an Axicon lens which produces a high intensity, highly localized, non-diffracting core with an extended depth of field[130]. In this way, a single

exposure of a photopolymer solution to the BB can produce long fiber structures through photo-polymerization caused by the high intensity BB core.

Hydrogel fibers have found numerous applications in the field of TE[246], giving rise to the development of multiple fiber fabrication techniques including microfluidic spinning[247], electrical and mechanical stretching[248], electrospinning[249], and melt spinning[250]. The ability to control fiber diameter is advantageous as it has been shown to affect cell morphology and cell alignment[251], and the ability to tune the fiber diameter has been demonstrated in the literature using different techniques[168-169]. For this reason, the ability to produce fibers of tunable diameters using the BB technique was tested by using the optical set up described in Section 5.2.2 and following the printing procedure described in Section 5.2.6.

Controlled fiber diameter

Figure 5.5(a-c) show acellular fibers fabricated following the procedure previously described with magnification $M=0.5$, 1, and 1.5, respectively. Fiber diameters were measured from the images at 10 random locations within the first 2mm from the attachment site and the averaged values were plotted against M in Figure 5.5(d). For $M=0.5$, 1, and 1.5, fibers obtained were 25 μm , 69 μm and 93 μm in diameter, respectively, i.e., fiber diameters increased as M increased. As the BB core was used for printing, the fabricated fiber diameters should mirror the BB core diameter.

Calculated BB core diameters, based on mathematical models described in Section 5.2.7, were also plotted in Figure 5.5(d) to compare against experimental fiber diameters and a strong relationship was observed where calculated BB core diameters were comparable in size to experimentally obtained fiber diameters i.e. fiber diameters could be accurately predicted and tuned appropriately through calculations. Theoretical calculation details are provided in Appendix C.

From the literature, using techniques such as microfluidics and wet spinning, the smallest fiber diameters fabricated were between 80 to 103 μm [168-170]. The smallest fibers printed in the current experiments was shown to be 25 μm , an approximately 75% improvement, thus demonstrating a significant improvement in printable fiber resolution using the BB printing technique. This printable fiber resolution can be further improved by reducing the M value. Although hydrogel fiber diameters fabricated using the electrospinning technique can go down to the nano-scale, the technique is not compatible with cells due to excessive heat generated during fabrication and cytotoxicity[169].

Figure 5.5. Acellular fibers fabricated with different values of M: (a) M=0.5, (b) M=1, (c) M=1.5. Green arrows indicate fiber locations. (d) Plots of M vs the experimental and theoretical values of fiber diameter. Strong correlation observed. Error bars represent standard deviation with n=10. (e) Fiber morphology when laser power was above threshold value, M=0.5. Fiber structures comprise a core surrounded by a cylindrical sheath. Green arrows indicate fiber core while red arrows indicate sheathing.

(f,h) Sheathed fibers produced repeatedly and consistently. (g) Magnified image of sheathed fiber clearly showing the regions produced by the BB core and the first ring. Scale bars: (a-e) 100 μ m (f, h) 500 μ m (g) 50 μ m.

Sheathing effect

In Figure 5.5(a-c), laser power was measured using a power meter (PM100D, Thorlabs), i.e., 50 μ W for $M=0.5$, 120 μ W for $M=1$, and 160 μ W for $M=1.5$, which ensured that only the BB core intensity was high enough to cause crosslinking. If the laser power used for fiber fabrication was higher than these values, the intensity of the first Bessel ring would be high enough to cause polymerization, resulting in the fibers being encased in a cylindrical sheath as shown in Figure 5.5(e-h). The BB profile was previously mentioned in the Literature Review Section 2.3. The dotted ring in Figure 2.9(a) indicates the region of the BB profile responsible for the fabrication of fibers encased in cylindrical sheaths. Magnified image of the sheathed fiber in Figure 5.5(g) clearly shows two distinct regions produced by the BB core and first ring. Sheathed fibers were produced consistently under constant fabrication parameters as illustrated in Figure 5.5(h). The fibers in Figure 5.5(a) were fabricated using $M = 0.5$, the same as the fibers in Figure 5.5(e), except that the laser power used for printing was increased to 100 μ W. The diameter of the fiber core in Figure 5.5(e) is comparable to the fiber diameter in Figure 5.5(a). This equivalent core diameter was also observed using $M=1$ and $M=1.5$ where fiber core diameters increased as M increased.

5.3.4 Cell encapsulation and viability

Cell-laden hydrogel fibers have shown promising results to be used in future for the production of patterned vascular networks in vitro as we progress towards more complex multicellular systems[32]. Cell-laden fibers have also been weaved into tissue fabrics with various spatial patterns in vitro, showing functionality during in vivo implantation[168]. Other than blood vessels, other types of fiber-shaped cellular

constructs exist in our bodies including neural bundles and fibrous muscle tissue, thus, microscale cell-laden fibers show promising applications for the construction of these linear tissues in vitro [169-170].

Cell encapsulation using BB

This chapter presents the first reported use of BBs for direct encapsulation of cells within hydrogels for TE applications that is not found in the literature. Figure 5.6(a-c) shows an example of 3 individual cell-laden fibers in a straight line array (10 total) separated by a distance of 500 μ m each, viewed from the top at different z-planes as illustrated in Figure 5.6(d). Cells can clearly be seen at all z-planes thus demonstrating homogenous cell encapsulation along the length of each fiber. The cell-laden fibers shown are floating freely in DMEM with one end firmly attached onto a coverslip. This printing technique allowed us to obtain customizable organized arrays of free-standing cell-laden GelMA fibers according to pre-designed array patterns. This capability is significant as it improves ease-of-handling and would allow us to control network architecture in future pre-vascularization applications.

Figure 5.6. Free-standing organized arrays of cell-laden fibers floating in medium. (a-c) Cell-laden fibers viewed at different z-planes showing cells encapsulated along entire length of fiber. (d) Schematic diagram of viewpoint and plane locations.

As cell density within the pre-polymer solution was increased between 2-10mil/ml, the density of cells within the fibers also increased (Figure 5.7). Thus, by varying the cell concentration in the pre-polymer solution we could control cell density within the fibers.

We did, however, observe that cell-laden fibers had a maximum printable length of around 1 cm, attributable to light attenuation and loss of laser power as the BB propagates through the cell suspension during fabrication. This is a significant limitation compared to other extrusion/microfluidic-based techniques where uniform cell-laden fibers of indefinite length can be produced[168-170]. Despite this perceived limitation, we should consider the intended application of the cell-laden fibers produced in this chapter. Our native vascular system does not consist of a single, long blood vessel of uniform diameter that runs throughout our body, but instead, it possesses a highly branched architecture of interconnected blood vessels with significant variations in diameter. With our current technology, we are unable to successfully replicate the complexity of our native vascular architecture, thus in order to produce pre-vascularized TE construct in vitro, we still rely on the ability of encapsulated ECs to form biomimetic vascular networks when encapsulated in a suitable ECM environment i.e. fibrin gel. With that in mind, the limited capability of the BB printing technique to produce cell-laden fibers more than 1 cm in length does not present a significant disadvantage when applied for pre-vascularization applications.

Figure 5.7. Tunable cell density within fibers by controlling cell density in pre-polymer solution. **(a-c)** Low cell density at 2mil/ml. **(d-f)** Medium cell density between 2-10mil/ml. **(g-i)** High cell density at 10mil/ml. 3 images were captured at different planes within the same fibers for each level of cell density. Cell density within fibers increased proportionally with increased cell density in pre-polymer solution. Arrows indicate fiber boundaries with encapsulated cells.

Cell viability

The available printing parameters used in conventional bioprinting techniques are often limited by cytocompatibility issues which result in poor cell viability and proliferation potential post-printing. Previous studies from the literature showed that for solid-freeform printing techniques (including inkjet and extrusion-based methods which are most commonly used in TE), increased dispensing pressure from 5psi to 40 psi resulted in a decrease in cell viability from 84.33% to 68.99%[251]. Printing temperature, when reduced from 30 °C to 22.5 °C (RT), caused a significant decrease in cell viability from >90% to <40%, thus requiring a temperature controlled nozzle for

optimal printing[252]. Exposure of the cells to shear stress during dispensing has been shown to be a major contributor to decreased cell viability – cell viability decreased to 76% when exposed to shear stress of more than 10kPa[253]. Other factors including needle geometry (tapered needle vs. cylindrical needle)[254], print material concentration[255], and the incorporation of nano-particles into the print material[256] were also shown to significantly affect cell viability, thus limiting the printable parameters that researchers can utilize to produce viable TE constructs.

At 3 days post-printing, Live/dead assay was performed to determine if exposure to UV in the current experiments affected the viability of encapsulated cells and to compare the current cell viability results with reports found in the literature. Figure 5.8 shows merged fluorescent images taken immediately after staining. Green represents live cells while red represents dead cells. Cells were counted using ImageJ software and the average cell viability from these images were calculated to be >95%, demonstrating that cell viability at day 3 was not significantly affected by the printing process. Compared to current literature, this result of >95% is considered a significant achievement.

Short wavelength UV light is known to cause long-term damage to cellular DNA which may cause a gradual change in phenotype that is undetected at the early stages. To understand the full effects of the exposure of cells to the UV light used in this experiment, characterization experiments at longer time points should be performed to detect any abnormal cell behaviour that may only appear at later stages.

Figure 5.8. Merged images of Live/dead assay performed at day 3 post-fabrication (Green: live, Red: dead). Scale bars = 50 μ m

Non-UV polymerization

There is growing research in the development of new photo-polymerization techniques which replace the use of short wavelength UV light with longer wavelength visible light[69,177,189,190], which should reduce cell damage upon exposure. However, this change would also require the use of new photoinitiators that are compatible with visible light. The conventional photoinitiator used by most research groups has been 1-[4-(2-hydroxy-ethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one (Irgacure2959) as it has proven to be cytocompatible at the concentrations frequently used for TE research. Recently, the use of other PIs have been suggested including 2,2-Azobis (2-methyl-N-(2-hydroxyethyl)propionamide) (VA086)[164] and lithium phenyl-2,4,6 trimethylbenzoylphosphine (LAP)[80,191] with published reports claiming superior qualities compared to the conventional Irgacure2959, including the absorption at higher visible light wavelengths. The absorption spectrum of LAP is found in Appendix D.

To determine the suitability of the 3 aforementioned PIs (Irgacure2959, VA086, and LAP) for use in bioprinting applications, the compatibility of each photoinitiator to print with 355nm UV light and 400nm visible light was tested and compared, as well as other factors including cell viability, solubility, gelling time, mechanical stability, and printing accuracy. All PIs were compatible with UV light (355nm) while only Irgacure2959 was incompatible with higher visible light (400nm) even at high dosage. Although there was negligible difference in cell viability at 3 days in vitro for all 3 PIs, it is known that cellular DNA damage caused by UV exposure may not show immediate effects, but the negative effects of damage may become evident only after long-term culture. LAP clearly outperformed the other two PIs in terms of solubility in water/PBS, gelling time, mechanical stability, and printing accuracy. A comparison

table for all 3 PIs tested is shown in Appendix E. Although LAP was shown to be superior compared to the other photoinitiators, in this dissertation, all printing was done using Irgacure2959 and not using LAP as the advantages of using LAP were only discovered towards the end of my candidature. However, in light of these results, and assuming that 400nm light is less damaging to cells in the long-term, LAP was shown to be the optimal photoinitiator and should be used for future printing. A more detailed study on the effects of using different PIs and light sources should be carried out in future work.

5.3.5 Cell proliferation and Guided cord-formation

In Chapter 4, it was found that HUVECs encapsulated within spatially confined hydrogel ECM were capable of self-assembly into endothelial cords. In this section, using the BB printing technique described in Chapter 3, fabricated cell-laden GelMA fibers were submerged in growth medium and subsequently stained and imaged with confocal microscopy to determine if endothelial cords were formed. The formation of endothelial cords in vitro is significant to the pursuit of pre-vascularization strategies in TE, as previously discussed in Section 2.4 of Chapter 2.

Cell proliferation

The ability for cells to proliferate indicates that the cells are adapting well to their environment and are healthy. It is also useful for TE applications where cells seeded or encapsulated within implantable scaffolds are expected to proliferate to sufficient numbers required for effective treatment. Figure 5.9 show images of DAPI stained nuclei of encapsulated cells within GelMA fibers at day 1, 3, and 5 post-printing. Cell density (cells/cm²) was observed to increase with time from 7×10^4 cells/cm² at day 1, to 2.2×10^5 cells/cm² at day 3, and finally 3.2×10^5 cells/cm² day 5. This shows that the cellular environment provided to the cells was conducive for their survival and growth.

Figure 5.9. Mask images of DAPI stained nuclei at day 1, 3, and 5 demonstrating increase in cell number. Cell density (cells/cm²) increased with time. Scale bars: 50μm for all images. Error bars represent SEM (n=4, *p<0.01).

Endothelial cord formation

Images of cell-laden GelMA fibers were captured at specified time points using confocal microscopy. Figure 5.10(a-c) show phase contrast images while Figure 5.10(d-f) shows merged 3D fluorescent images captured at day 1, 3, and 5 respectively. Blue represents cell nuclei while red represents actin. Figure 5.10(g-i) shows cross sectional views of the 3D images of Figure 5.10(d-f), respectively. Three cross-sectional slices were captured for each 3D image. Cross-sectional slice locations are drawn in the 3D images and are labelled according to their planes (x-y, x-z, and y-z). From the cross-sectional images we see that at day 1, there is hardly any connection between individual cells. At day 3, we begin to see some connections made between cells, but the formation of confluent endothelial cords was not observed. At day 5, cells were connected into a confluent endothelial cord structure

surrounding an acellular region as seen by the dark regions pointed out by the arrows in Figure 5.10(i). This shows the successful self-assembly of HUVECs into endothelial cords when encapsulated within GelMA fibers using the BB printing technique.

Figure 5.10. Phase contrast and fluorescent confocal images taken at day 1, 3, and 5. **(a-c)** Phase contrast images. **(d-f)** 3D fluorescent images. **(g-i)** Cross-sectional images corresponding to slices indicated in the 3D images. White arrows in **(i)** point to dark regions without cells indicating endothelial cord formation. Scale bars: 50µm for all images.

In a previous study found in the literature, cells encapsulated within collagen filled PDMS channels did not migrate toward the non-adhesive PDMS walls[103]. From Figure 5.10 in this study, cells were found at the fiber edges on day 1. This could have occurred due to the free-standing nature of the fabricated cords where ECs were exposed to cell medium from all directions, causing encapsulated ECs to migrate toward the fiber edges and closer to the nutrient source, thus allowing direct

exposure of the cells to the surrounding growth medium without diffusion through the GelMA.

Perfusability

Although confocal images showed a clearly defined circular actin cross-section formed by day 5 HUVECs, it has not been demonstrated to be hollow and perfusable. From past literature, HUVECs encapsulated within collagen/GelMA channels were shown to self-assemble into non-hollow cords which contain dense gel within the lumen[103,106]. Given the similarities in material choice and methods, the cords fabricated in this chapter should also not be hollow and should contain gel within their lumen, although further staining results need to be done for confirmation. However, reports have shown that despite the cords being non-hollow, in vivo implantation of these non-hollow cords enhanced the formation of mature blood-perfusable vasculature with controlled architecture through vasculature infiltration from the host and cellular remodelling of the ECM [36,104].

5.4 Summary

In this chapter, the BB printing technique was used for direct encapsulation of live HUVECs within GelMA fibers, which has never been reported in the literature, for TE and pre-vascularization applications. First, the mechanical properties of GelMA were characterized and shown to be tunable based on degree of methacrylation and concentration. Acellular fibers were then printed using the BB technique and showed accurate tunability of fiber diameter based on mathematical models. The printable fiber resolution of 25 μm was a significant improvement compared to conventional fiber fabrication techniques. Cells were then directly encapsulated within customizable arrays of GelMA fibers through in situ photopolymerization and cells were shown to be evenly distributed within the fibers with controllable cell density.

The maximum printable fiber length of 1 cm presented a limitation compared to other fiber fabrication techniques, however it does not pose a significant disadvantage considering the intended application which is for pre-vascularization of TE constructs. The BB printing technique displayed superior cytocompatibility compared to conventional printing systems based on cell viability quantification post-printing. The BB technique was also compatible with visible light for photopolymerization instead of UV light, thus potentially improving the cytocompatibility further although definitive data was not presented in this chapter. Several days after encapsulation within GelMA fibers, HUVECs were shown to proliferate with time and self assembled into endothelial cords in vitro. The successful printing of self-assembled free-standing endothelial cords could provide added flexibility and control over subsequent procedures in the pre-vascularization process i.e. co-culture with parenchymal cells, control over cord maturation process, direct seeding of perivascular cells on the outer walls of the cord etc. which would be useful in the field of TE. The printed endothelial cords can be harvested and co-cultured with other cell types in future to produce 3D vascularized tissue constructs in vitro which would bring us a step closer to successfully engineering complex tissues and organs.

6. Conclusions and future work

6.1 Conclusions

6.1.1 Chapter 3: Laser-based 3D printing using Bessel Beams

A laser-based additive manufacturing technique using BBs which possess several unique properties, such as non-diffraction and high depth-of-field, which distinguishes it from conventional Gaussian beams used in commercial printing systems, was investigated. Using the BB technique, the ability to customize cross-sectional shape, size, and length of fabricated tubular constructs was demonstrated to replicate the various tubular organs in our bodies which vary in dimensions. As an example, anatomically relevant tubular construct which fit the dimensions of a small-diameter vascular graft were fabricated. The technique was shown to reduce fabrication time by more than two-fold when compared to existing printing techniques. High aspect ratio tubular structures were successfully fabricated without the concurrent fabrication of supporting material which is required by conventional printing techniques. The hydrogel used for printing (PEGDMA) had mechanical properties comparable to articular cartilage, thus making it suitable for future applications in articular cartilage regeneration. The BB technique's capability to fabricate hydrogel scaffolds with built-in microchannels and controlled grid spacings efficiently without the layer-by-layer processing as demanded by conventional printing techniques was also demonstrated. Compared to extrusion-based techniques, a high print resolution was achieved (80 μm) that could be reduced further through optical manipulation (demonstrated in Chapter 5). With the addition of fluorescent microbeads into the pre-polymer solution, pronounced deformation of the fabricated constructs was observed, thus presenting a limitation to the printing technique.

6.1.2 Chapter 4: Environmental effects on self-assembled endothelial network formation in hydrogels

HUVECs were encapsulated within hydrogel ECM with varying properties and their effects on self-assembled HUVEC networks were characterized. It was found experimentally that at the appropriate concentrations, collagen gels were able to induce HUVEC sprouting and support cell viability. However, cells fully encapsulated in 3D collagen ECM remained rounded and no sprouting was observed except at the gel-coverslip interface. Confocal imaging confirmed that sprouts formed at the gel-coverslip interface were non-lumenized, a result that is contrary to existing reports in the literature thus providing clarity on the subject. It was also found that spatial confinement was necessary for endothelial cord formation in collagen gels. Endothelial sprouting was observed in all directions for HUVECs encapsulated in fibrin gel, thus indicating its superior vasculogenic potential. This finding suggests that fibrin gel is recommended for use as bulk ECM material for the fabrication of complex vascularized tissue in future.

6.1.3 Chapter 5: Cell-laden fiber fabrication for endothelial cord formation using Bessel Beams

Knowledge on the capabilities of the BB printing technique from Chapter 3 and information gained in Chapter 4 were combined to successfully demonstrate the promising potential of the BB printing technique for applications in TE. Firstly, the mechanical properties of the cytocompatible print material (GelMA) was shown to be tunable which is useful for future research and the replication of a variety of soft tissue. GelMA fibers fabricated using the BB technique were also shown to be tunable and accurately predictable based on mathematical models; a significant result considering that fiber diameter has been shown to affect cell morphology and alignment. The printable fiber resolution was found to be 25 μm , a significant improvement from other fabrication techniques including microfluidics and wet

spinning. Live cells were successfully encapsulated with even cell distribution within GelMA fibers which were organized in customizable arrays for ease of handling and potential control of network architecture in pre-vascularization applications. Cell density within the fibers was also shown to be easily controllable. However, the maximum printable length of 1 cm for cell-laden fibers printed using the BB technique presents a significant limitation compared to existing techniques, however, for pre-vascularization applications, this perceived limitation does not present a significant disadvantage. Superior cytocompatibility of the BB printing technique was demonstrated with live/dead staining results showing >95% cell viability; a significant result compared to conventional extrusion and inkjet-based systems. Cells were also observed to proliferate with time indicating that they were healthy and adapting well to their conducive cellular environment post-printing. Based on proof-of-concept experiments conducted where non-conventional photoinitiators were used, the BB technique was compatible with the use of visible light instead of UV for polymerization which should result in further improvement to the cytocompatibility, however the experiments conducted were rudimentary and more detailed experiments are required to produce definitive data. Finally, in a culmination of all the work presented in this dissertation, HUVECs encapsulated in gelMA fibers fabricated using the BB printing technique were shown to successfully self-assemble into endothelial cords in vitro, thus confirming and solidifying the printing technique's applications in TE.

6.2 Future work

As mentioned in 'Chapter 3.4 Discussion', there are several drawbacks to using the BB technique for bioprinting applications including wall thinning and limited design complexity. Future work should include a balanced evaluation of how the proposed

BB printing technique compares to established, commercially available bioprinting systems in order to establish it as a viable alternative to current technology.

Properties to be evaluated include:

- Limitations in structural design complexity.
- Ability for multi-material, multi-cellular construct fabrication.
- Difference in fabrication time for varied construct designs.
- Long term effects on cell phenotype/genotype from UV exposure.
- System flexibility in terms of tuning the print resolution in real time.
- Structural non-conformity to original design.

Figure 6.1. Future work. **(a-b)** Free-standing endothelial cords are directly seeded with perivascular cells (pericytes/SMC) to more accurately recapitulate the structure of in vivo blood vessels. **(c-d)** Fabricated cords are encapsulated within cell-laden fibrin gel and supplemented with medium to induce angiogenic sprouting, resulting in a fully vascularized 3D tissue construct with controlled architecture.

The end goal for tissue engineers is to produce functional, biomimetic, vascularized tissue constructs suitable for the treatment of damaged tissue. Figure 6.1 illustrates future procedures using the BB printing technique that may be implemented to achieve this ultimate goal. The successful fabrication of in vitro endothelial cords in

patterned arrays (Figure 6.1a) has been demonstrated previously in Section 5.3.5. Perivascular cells (PCs) such as pericytes, smooth muscle cells (SMCs), and fibroblasts play vital roles in the formation[219–221], maturation[222–224], and function[225–227] of native blood vessels. Using the BB technique to fabricate free-standing endothelial cords allows direct seeding of pericytes on the outer wall of the cords which mimics the architecture of native blood vessels (Figure 6.1b). After cord maturation, the cords can be encapsulated within fibrin gel laden with ECs and parenchymal cells of the user's choice i.e. neurons to simulate blood-brain barrier (Figure 6.1c). After supplementing with medium, vasculogenesis or angiogenic sprouting should occur (as observed in current experiments) within the cell-laden fibrin gel resulting in a fully vascularized 3D tissue construct.

Publications

Journal articles:

- **Liew, Andy Wen Loong**, and Yilei Zhang. "In vitro pre-vascularization strategies for tissue engineered constructs – Bioprinting and others." *International Journal of Bioprinting* (2017) **3** 3-17.
- **Liew, Wen Loong Andy**, and Yilei Zhang. "Laser-based fabrication of 3D hydrogel constructs using bessel beams." *Bioprinting* (2018) **9** 44-51.
- **Liew, Wen Loong Andy**, and Yilei Zhang. "Cell-laden gelatin methacryloyl fibres fabricated using bessel beams for controlled endothelial cord formation." *Biomedical Physics & Engineering Express* (2018) **4** 045009.

Conference Paper:

- **Liew, Andy Wen Loong**, and Yilei Zhang. "Fabrication of lumenized vascular tubes for tissue engineering applications." *Proceedings of the 2nd International Conference on Progress in Additive Manufacturing* (2016).

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Appendix A: UV dose calculations

	M=0.5	M=1	M=1.5
Fiber diameter(μm)	25	69	93
Area (μm^2)	491	3739	6793
Area(cm^2)($\times 10^{-6}$)	4.91	37.39	67.93
Power(μW)	50	120	160
Power(mW)	0.05	0.12	0.16
Intensity(mW/cm^2)	10186	3209	2355
Exposure time(s)	5	5	5
Dose(mJ/cm^2)	50929	16046	11777

Appendix B: Absorption spectra of Irgacure2959 photoinitiator

Graph acquired from supplier information (Ciba).

Appendix C: BB core diameter calculation

$$\theta \approx \left(\frac{\pi}{2} - \frac{\alpha}{2}\right) \left(\frac{n_a}{n_m} - 1\right) \quad (1)$$

$$r_b = \frac{2.4048}{k \sin \theta} \quad (2)$$

$$M = \frac{f_2}{f_1} \quad (3)$$

$$r'_b = Mr_b \quad (4)$$

$$r'_b = M \frac{2.4048}{k \sin \theta} \quad (5)$$

Common variables:

$$n_m = n_{air} = 1$$

$$n_a = 1.5$$

$$\alpha(deg) = 179^\circ$$

$$\alpha(rad) = \frac{179 \cdot \pi}{180} = 3.124$$

$$\text{From (1): } \theta \approx \left(\frac{\pi}{2} - \frac{3.124}{2}\right) \left(\frac{1.5}{1} - 1\right) = 0.004363\text{rad}$$

$$\lambda = 355 \times 10^{-9} m$$

$$k = \frac{2\pi}{\lambda} = \frac{2 \cdot \pi}{355 \times 10^{-9}} = 17699113.5 m^{-1}$$

Configuration 1

$$f_1 = 1000 mm$$

$$f_2 = 500 mm$$

$$\text{From (3): } M = 0.5$$

$$\text{From (5): } r'_b = 0.5 \frac{2.4048}{17699113.5 \sin 0.004363} = 15.6 \mu m$$

$$d_b = 2r'_b = 15.6 \times 2 = \mathbf{31.2 \mu m}$$

Configuration 2

$$M = 1$$

$$\text{From (5): } r'_b = \frac{2.4048}{17699113.5 \sin 0.004363} = 31.1\mu m$$

$$d_b = 2r'_b = 31.1 \times 2 = \mathbf{62.2\mu m}$$

Configuration 3

$$f_1 = 500mm$$

$$f_2 = 750mm$$

$$\text{From (3): } M = 1.5$$

$$\text{From (5): } r'_b = 1.5 \frac{2.4048}{17699113.5 \sin 0.004363} = 46.7\mu m$$

$$d_b = 2r'_b = 46.7 \times 2 = \mathbf{93.4\mu m}$$

Appendix D: Absorption spectra of LAP photoinitiator

Molar absorption of LAP (solid line) and cleavage products (dashed line). Adopted from ref [191].

Appendix E: Photoinitiator comparison table

	Irgacure2959	VA086	LAP
Compatible with 355 nm laser	Yes	Yes	Yes
Compatible with 400 nm laser	No	Yes	Yes
Cytocompatibility at day 3	Negilible difference		
Solubility in PBS	Poorest	Medium	Best
Gelling time	Medium	Poorest	Best
Mechanical stability of printed construct	Medium	Poorest	Best
Print accuracy	Medium	Poorest	Best

Appendix F: Collagen gelling protocol

Alternate Gelation Procedure for Corning Collagen I HC, Rat tail

- 1.0 Place on ice the following:
 - 1.1 Corning Collagen I HC, rat tail
 - 1.2 Sterile 10X phosphate buffered saline (10X PBS)
 - 1.3 Sterile dH₂O
 - 1.4 Sterile 1 N NaOH
- 2.0 Determine the final volume of Corning Collagen I HC, rat tail, solution to be used and the desired final collagen concentration.
- 3.0 Place on ice a sterile tube of sufficient capacity to contain the final volume of Corning Collagen I HC, rat tail.
- 4.0 Perform the following steps using aseptic technique in a Class 100 Hood.
 - 4.1 Add to the tube the following volume of 10X PBS:
$$\frac{\text{Final Volume}}{10} = \text{mL 10X PBS}$$
 - 4.2 Calculate the volume of Corning Collagen I HC, rat tail, to be used (do not add to the tube until step 4.6):
$$\frac{\text{Final volume} \times \text{Final collagen concentration in mg/mL}}{\text{Concentration in bottle (see lot specific spec. sheet)}} = \text{volume collagen to be added}$$
 - 4.3 Add to the 10X PBS the following volume of sterile ice cold 1 N NaOH:
$$(\text{volume collagen to be added}) \times 0.023 \text{ mL} = \text{volume 1 N NaOH}$$
 - 4.4 Add to the 10X PBS/1 N NaOH the following volume of sterile ice-cold dH₂O:
$$(\text{Final volume}) - (\text{Volume collagen}) - (\text{Volume 10X PBS}) - (\text{Volume 1 N NaOH}) = \text{Volume dH}_2\text{O to add}$$
 - 4.5 Mix the contents of tube and hold in ice.
 - 4.6 Add the calculated volume of Corning® Collagen I HC, rat tail, and mix. Leave on ice until ready for use.
- 5.0 The Corning Collagen I HC, rat tail, solution can be used immediately or held on ice for 2-3 hours.
- 6.0 When ready to use, aseptically deliver the solution into the cell culture device and allow to gel at 37°C for 30 minutes.

NOTE: For more details on Corning Collagen products and technical resources please visit support page at www.corning.com/lifesciences