Miniaturized 3D fibrous scaffold on stereolithography-printed microfluidic perfusion culture

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**MINIATURIZED 3D FIBROUS SCAFFOLD ON
STEREOLITHOGRAPHY-PRINTED MICROFLUIDIC
PERFUSION CULTURE**

NG FENG LIN

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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STEREOLITHOGRAPHY-PRINTED MICROFLUIDIC
PERFUSION CULTURE**

NG FENG LIN

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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

2018

Statement of Originality

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

24/08/2018

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Date

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Supervisor Declaration Statement

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

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Authorship Attribution Statement

This thesis contains material from a paper published in the following peer-reviewed journal where I was the first author.

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The contributions of the co-authors are as follows:

- A/Prof Tan provided comments on the project directions, ideas and edited the manuscript drafts.
- I have prepared the manuscript drafts and design of the experiments.
- I have performed all the laboratory work at the School of Materials Science and Engineering and SIMTech. I have also analyzed the data.
- All scaffold preparation, scaffold characterization, and cell studies, were conducted by me in the School of Materials Science and Engineering.
- Ms. Ong Yee Oon was involved in the optimization of 3D aligned scaffold as an intern in SIMTech and FYP student in NTU.
- Dr. Chen Huizhi provided advises on cell studies
- Dr. Le Quan Ngoc Tran provided advises on the Wilhemly plate method studies.
- Dr. Cao Ye provided advises on human aorta smooth muscle cells nature and its cell culture studies
- Dr. Tay Bee Yen provided advises on the design of electrospinning setup

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Abstract

Cells cultured on 2-Dimensional (2D) and 3-Dimensional (3D) substrates generally display significant differences in terms of cell shape, proliferation, morphogenesis and differentiation. While 3D substrates provide better interaction with cell-cell, cell-ECM or cell-soluble factors, tissue construct greater than 200 μm will experience involuntary cell death, e.g. necrosis within the construct. Such an involuntary cell death can be prevented by providing sufficient perfusion through the construct.

The objective of this project is to develop a miniaturized 3D micro-perfused system where cells are provided with a highly relevant micro-environment by adopting the use of 3D scaffold and local perfusion. The 3D fibrous scaffold used in this study was to recapitulate the fibrillary and porous nature of the extracellular matrix of a native tissue. At the same time, local perfusion was provided to ensure sufficient exchange of nutrients and metabolites. The proposed setup is much preferred over the conventional 2D static culture since it better mimics the *in vivo* micro-environment, especially for highly perfused organ such as liver.

To realize the idea and design of the micro-perfused platform, an Electrospinning auxiliary jig has been designed for the collection of 3D aligned or random scaffold. For a demonstration, 3D random scaffold has been embedded on a Stereolithography-printed perfused platform. A one-step Stereolithography process has been developed where the 3D fibrous scaffold was embedded without any tedious secondary assembly processes. This is the first instance where a miniaturized 3D fibrous scaffold has been embedded on a Stereolithography-printed perfused microfluidic platform.

Huh7.5 hepatocellular carcinoma cells were used as model cells for this platform since liver hepatocytes experienced similar perfused micro-environment. Preliminary cell studies revealed that Huh7.5 presents sufficient cells viability and proliferation on the micro-perfused system. Cells infiltration were found to be better than a static culture due to the presence of fresh medium and the gene expression of Albumin and CYP3A cytochrome

was found to be much upregulated as compared to its static counterpart.

Taken together, the newly developed device has proven to be a physiologically-relevant platform for the maintenance of liver hepatocytes. While the embedded scaffold for this instance was of random nature since the extracellular matrix (ECM) of liver do not exhibit any preferential alignment; the use of 3D aligned fibrous scaffold on the platform is possible. The device developed in this study has proved to be highly versatile and tailorable that suits the local *in vivo* micro-environment of various organs and tissues to be studied in future.

Lay Summary

Drugs developed by Pharmaceutical companies usually involved tedious regulatory scrutiny prior to approval. An average drug expenditure is projected to be \$1 billion in excess before it can be taken to the market. A recent study by the Journal of Health Economics reported that there is only approximately 1 in 1,000 potential drugs is being taken to the human clinical trials after pre-clinical testing in the United States. Almost 9 out of every 10 drugs failed the human testing phase. Such losses can be mitigated should there be a highly relevant tissue model that mimics the human tissues.

Drug failure during the clinical trial phases were largely due to the irrelevancy of pre-clinical studies which uses conventional tissue culture plates and animal models. Conventional tissue culture plates were mainly a pre-treated Polystyrene substrate (TCPS) that were of 2-Dimensional (2D) format. While the animal model studies were not a good representation of drug metabolism in human. Thus, there is an imperative need to develop a platform that better mimics the actual human environment so that preliminary drug screening results are more relevant.

In this study, a micro-perfused platform has been developed to better model the local environment of tissues and organs. The platform consists of a miniaturized piece of fibrous substrate which mimics the actual environment where cells reside in the body. In addition, perfusion flow on the substrate has been introduced to better mimic the fluid exchange in the body which is often lacking in the conventional 2D static culture. The resultant platform is therefore a more biologically relevant environment which consist of the fibrous nature and perfusion fluid flow.

Liver cancer cell line, Huh7.5, was used as model cells in this study to determine the relevancy of the developed platform. Preliminary cell studies suggest that Huh7.5 liver cancer cells were better maintained in the newly developed platform. Upregulation of Albumin and Cytochrome 3A, an important enzyme for the metabolism of drugs were seen on the newly developed platform.

Taken together, the newly developed micro-perfused platform has proven to be a relevant model for the use of early screening of drug studies. The developed platform may not necessarily be seeded with liver cancer cells, as it could be used for other cell types such as human aorta cells where perfusion of such cells is necessary for its functionality. The versatility of the developed platform will therefore suit various organs and tissues to be studied in future.

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*I dedicate this
thesis to
my family and
hubby, Garek Ang
for their love,
support &
encouragement*

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Abbreviations

2D	Two-Dimensional
3D	Three-Dimensional
SLA	Stereolithography
FDM	Fused Deposition Modeling
3DA	Three-Dimensional Aligned
3DR	Three-Dimensional Random
CFD	Computational Fluid Dynamic simulation
PDMS	Polydimethylsiloxane
PCL	Polycaprolactone
PLGA	Poly(lactic-co-glycolic acid)
IPA	Isopropyl Alcohol
ETOH	Ethanol
SEM	Scanning Electron Microscopy
Huh 7.5	Human hepatocellular carcinoma cell line
hSMCs	Human aorta smooth muscle cells
qPCR	Quantitative real time polymerase chain reaction
ECM	Extracellular matrix
α -SMA	Alpha smooth muscle actin
MHC	Smooth muscle myosin heavy chain
TCPS	Tissue Culture Polystyrene (2D monolayer)
3DR/Static	Three-Dimensional random fibrous scaffold on static culture
3DR/Perfused	Three-Dimensional random fibrous scaffold on perfused culture

Chapter 1

Introduction

Cells cultured on 2D and 3D substrates were reported to display significant differences in terms of cell shape, proliferation and its phenotypic functions. With the development of micro-/nano technologies, nanofibrous construct that mimics the native extracellular matrix is made possible. However, as the tissue engineered construct gets thicker than 200 μ m, necrotic region starts to form within the tissue. In order to overcome the involuntary cell death and provide thick functional tissue construct for various tissue engineering applications, a micro-perfusion culture is highly desirable. This chapter introduces the current need in thick engineered tissue construct and its means in fabricating a functional one.

1.1 Background

1.1.1 Needs and Progress in Tissue Engineering

Tissue engineering has proven to be a solution to repair, restore and augment functions of the injured or diseased counterpart *in vivo* [1]. Tremendous effort has been put together to develop solutions for various needs in tissue engineering, such as the discovery of induced pluripotent stem cells (iPSCs) that paved its way for personalized medicine [2, 3], the effect of substrate stiffness on stem cells modulation [4, 5] and the development of scaffolds and devices that led to the fabrication of better biomimetic tissues [6-11]. Presently, in order to have full recapitulation of tissue or organ, a high density of multiple functional cell types is necessary. While the major study in this field involves understanding the effect of intricacies of cell micro-environment on various level, the conventional tissue engineering approach based on scaffolding or 2D monolayer are limited in their capacity to produce 3D tissue construct suitable for implantation [12].

Reports have shown that cells seeded on 2-Dimensional (2D) and 3-Dimensional (3D) substrates generally display significant differences in terms of cell shape, proliferation, morphogenesis and its phenotypic functions. Figure 1.1 presents some work on primary cells cultured on 2D monolayer and 3D culture, clearly showing that the cell morphology of hepatocytes and fibroblasts is distinctly different when cultured on a 3D substrate, with the morphology taking after its native tissue better [5, 13]. Essentially cell fate is determined by its interaction with its surroundings such as ECM, cells and other soluble factors. Henceforth, a 3D culture model that provide a better physiological equivalent substrate for drug testing, disease modelling or tissue development is very much desired.

Figure 1.1 Cellular morphology of cells cultured on 2D versus 3D substrate [14, 15]

While there are several commercially available 3D culture system such as Organovo™, InSphero, CN Bio Innovations and AIM Biotech start-ups [16]. They were mainly using hydrogels or the hanging drop method to form cluster of cells. Such a method although has been widely studied and proven cells-viable [17, 18], there is still a lack of fibrillary porous scaffold on these commercial platform. A biomimetic scaffold with fibrillary and porous nano-features are highly desirable since it mimic the natural ECM, provide physical and chemical cues for cell growth it also promote essential cellular processes [19]. Hence the addition of a bio-mimicking construct on a micro-platform would be highly beneficial and provide greater relevancy as a drug testing platform.

With the recent development of micro-/ nanotechnology such as electrospinning or 3D printing, the reconstruction of fibrous architecture that mimics the native extracellular matrix is made possible. Electrospinning, a facile nanofibers fabrication method which uses electric potential to regulate the collection of fibers on a targeted substrate, has been used widely to form biomimetic scaffold [20, 21]. The fibers formed are usually continuous, either randomly oriented or aligned fibers by using a stationary or rotating collector respectively. Unconventional electrospinning techniques such as the water bath collector

enable the formation of thick 3D random oriented scaffold [22]. These scaffolds are made up of continuous fibers, with high porosity and spatial interconnectivity which address the limitation of poor cells and reagent infiltration on 2D substrates [19, 22].

3D printing technologies such as bioprinting, fused deposition modeling (FDM) or stereolithography (SLA) have been extensively used for various applications in tissue engineering [12]. 3D bioprinting technology uses bioink to co-extrude cells with precise control over its composition and spatial distribution to deliver a 3D cell-laden construct. The long term stability of the bioprinted construct is dependent on the secondary cross-linking process. An initial high density of cells is necessary to ensure post-printing functionality, it is also important to ensure that cells do not experience excessive shear stress during the printing process [8].

FDM involves the melting of a thermoplastic polymer through a heated nozzle followed by deposition on a movable platform to form a 3D construct. The process is considerably simple and low cost, however there are only limited availability of thermoplastic material and the mechanical properties is relative weaker due to poor inter-layer connection [23]. The typical printing resolution for FDM falls within the range of 50 to 200 μm and it is used widely in bone tissue engineering due to its limited material selection [23, 24].

Stereolithography (SLA) on the other hand uses UV light to polymerize the liquid monomer one layer at a time which then build into a three-dimensional construct. The monomer used is commonly an acrylic-based resin that polymerized into polymer upon UV activation. After photo-polymerization, the pattern of the desired structure is printed on an upward moving platform which served as a holder for the subsequent layer. The general resolution for SLA falls in the range of 20 to 50 μm and is the preferred choice due to better resolution and stronger mechanical properties [23]. However, the process is considerably more expensive than most of the other 3D printing technologies.

While each of the approaches seems promising, electrospinning and stereolithography clearly provides greater ease and flexibility in achieving mature cell-laden grafts that is comparable to their native counterpart. However, as the 3D tissue construct gets thicker (e.g., more than 200 μm) necrosis occurred within the tissue [25]. The involuntary cell death can be prevented with a constant exchange of nutrients and cell metabolites on a perfusion platform such as bioreactors or microfluidic perfusion device [26, 27]. The perfusion platform should therefore support 3D tissue development, cell viability and maintain its function within the tissue.

1.1.2 Perfusion based culture

The critical aspect of engineering functional tissue lies in providing sufficient mechanical and biological support for cells while allowing good mass exchanges and blood circulation within the tissue. Often oxygen has low solubility and it diffuses through only 100 to 200 μm , hence limiting the survival of cells in large anatomically relevant graft [26]. The fundamental criteria for engineering functional tissue thus include: (i) Providing a sufficient size and mechanical properties of engineered tissue for the handling and viability under *in vivo* conditions; (ii) The biomechanical function of the engineered tissue should improve gradually until the function of normal tissue has been restored; (iii) After implantation, the engineered tissue graft should not elicit host responses [25, 26].

Cells cultured *in vitro* tend to retain its phenotypic function under condition that mimics the native *in vivo* environment. Such environment can be achieved through the development of 3D scaffold, incorporation of bioreactors and growth factors to deliver anatomically functional tissue [25]. Studies have shown that cellular apoptosis and necrotic regions within 3D engineered construct is prominent under static culture due to insufficient mass transport of oxygen, nutrients and wastes [26, 28, 29]. In this aspect, a perfusion-based culture would mitigate the mass transport limitations and provide a controlled micro-environment for the development of 3D tissue construct.

Figure 1.2 illustrates the schematic on examples of flow perfusion cultures. (Left) presents a typical large perfusion bioreactors which require large amount of cells, material and reagent [30]. (Right) presents a cell perfusion culture system on microfluidic platform which requires lesser cells, material and medium [31]. Each of the culture present its pros and cons; in the current study the microfluidic perfusion culture is the preferred choice since minimal cells and reagents is needed for various analysis.

Figure 1.2 Examples of perfusion-based culture setup. (Left) A typical perfusion-based bioreactor. (Right) A microfluidic perfusion culture.

Microfluidics cell culture platform is emerging as an important tool for the discovery of drugs and biological application research since they can reduce experimental time and reagent volume hence translating to higher experimental throughput [32, 33]. The typical time taken for drug candidate to be approved successfully as new drug takes more than a decade. Large amount of material and resources are needed to put through the application process some of which include the need to analyze drug candidate in a more efficient manner. This presents an emerging and exciting opportunity for microfluidic cell culture platform to be developed as a new tool that expedite drug discovery and development process [32].

The microfluidic platform have also found its application in the culturing of cells where such microscale level allow precise study and control of the extracellular micro-environment [31]. The reduction of culture volume in a microfluidic device is beneficial when large amount of cells or culture media could be relatively expensive for some biological research, example stem cell differentiation studies. In addition, the small volume of a microfluidic cell culture chamber makes it possible to study the spatial effects on single cell.

The additional advantage of microfluidic cell culture is the flexibility to incorporate auxiliary attachment to the culture platform. The auxiliary attachment in this aspect could be a perfusion pump where a continuous flow of media was introduced to the cells cultured within the chamber. Such a perfusion system on a microfluidic platform is considerably attractive for highly perfused organs such as liver or kidney; in addition, the limitation in providing sufficient transport of oxygen and nutrients can be addressed too.

Although some of the microchips are commercially available for non-cell based *in vitro* studies, it is important to incorporate cells in particular liver hepatocytes for drug testing applications. The primary liver hepatocytes contain a broad range of enzymes, hepatic transporters and other differentiated function needed for the metabolism of drugs. The main enzymes that contributed to metabolism belong to the cytochrome P450 group, where the maintenance of these functions would ensure that drugs are metabolized in a similar manner to the *in vivo* condition. Therefore the development of a micro-liver perfused platform in this study not only facilitates multiplexing for drug screening at the same time the microfluidic hepatocyte chip would also be better in predicting the drug efficacy that provides a more predictive *in vivo* cellular responses [31].

1.2 Objectives and Scope

The general objective of this study is to develop a miniaturized 3D biomimetic scaffold housed within a perfused microfluidic cell culturing chamber. This can be achieved through a combination of scaffolding, 3D printing and microfluidics perfusion technology. The perfusion flow on a microfluidic system was thought to enable a controllable distribution of oxygen and nutrients to metabolically active cells and shear stress at a cellular resolution. Such a platform is highly desirable for tissue types that are highly perfused *in vivo*, example liver and kidney. In this aspect the microfluidic hepatocyte chip developed would enable multiplexing for drug testing where the perfusion system would better mimic the *in vivo* micro-environment. With the current development of 3D printing technologies, the design iteration of features on the microfluidic chip is made with greater ease and agility.

The specific objectives are:

1. Development of electrospinning counter-electrode for fabrication of 3D aligned and random scaffold to be incorporated into the microfluidic cell culturing chamber
2. Development of a one-step stereolithography (SLA) process for the fabrication of microfluidic perfusion culture
3. Determine the cell viability and phenotypic maintenance on miniaturized 3D perfused microfluidic chip and 3D aligned fibrous scaffold

1.3 Dissertation Overview

The overall framework of the thesis addresses the need to develop a miniaturized 3D micro-environment that better mimics its natural *in vivo* counterpart. Miniaturized 3D fibrous scaffold embedded on a perfused microfluidic platform has been proposed in this study. A perfused 3D culture is much preferred over the conventional 2D static culture since it better mimics the *in vivo* micro-environment especially for cells that reside in close proximity within the microvascular network, such as liver or kidney. Multiple design iteration of the 3D microfluidic culture platform is made easier using the current 3d printing technology.

The overall study of the dissertation can be broken down into 3 areas:

Section 1: Develop an electrospinning counter-electrode for 3D aligned and random scaffold

3D biomimetic scaffolds with fibrillary and porous nano-features are highly desirable since the porous nature promote essential cellular exchanges as compared to 2D substrates [19]. While several nanofabrication techniques has been developed for various purposes, some processes have been adopted for the fabrication of scaffold [20].

Electrospinning in this case is deemed as the favorable method for it produces nanofibrous structure that is similar to the ECM architecture [19, 20, 22]. However, conventional electrospinning is limited to 2D substrate that is random and dense. Thus, in this study a flexible counter-electrode has been designed to collect 3D fibrous aligned and random scaffold in an optimized water bath collector. The rotating counter-electrode has demonstrated great flexibility in collecting fibres on any platform such as a micro pin for microvascular demonstrator. Physical characterization has been performed for both 3D random and 3D aligned scaffold.

Section 2: Design of a one-step stereolithography (SLA) process for microfluidic perfusion culture

With the recent development of 3D additive manufacturing, the rapid design iteration and fabrication of microfluidic chip is well facilitated. Among the 3D additive manufacturing techniques, stereolithography was deemed as the preferred option for the fabrication of perfused microfluidic chip since it has higher resolution to resolve features on the microfluidic platform; the mechanical properties of the liquid resin used in this process is significantly superior.

Conventional assembly of the microfluidic platform is considerably tedious and inefficient since it requires pre-treatment of microfluidic chip followed by the bonding process. Herein we present a one-step stereolithography process that embeds the 3D fibrous scaffold and at the same time obviates the need for secondary bonding processes. The physical characterization and fluid flow computational studies have been performed on the microfluidic device to determine the optimal condition and flow rate for the perfusion of hepatocyte culture.

Section 3: A demonstration of cell viability and phenotypic maintenance on miniaturized 3D perfused microfluidic chip and 3D aligned fibrous scaffold

In general, cells when placed under suitable conditions is capable of remodeling their micro-environment and form functional tissue unit. Liver, a highly perfused organ that perform function such as the metabolism of drugs may be recapitulated adequately on a perfused microfluidic platform. Human hepatocellular carcinoma cells (Huh-7.5) were used in this study as model cells to determine the functionality of the designed miniaturized perfused microfluidic platform. The viability, proliferation and key liver functions will be examined.

Reports have shown that the effect of nanofibrous surface topographical cues were beneficial in maintaining cells phenotype [31]. Therefore, the maintenance of vascular smooth muscle cells phenotypes was studied on 3D aligned fibrous scaffold as described in section 1.

Figure 1.3 Overview of proposed research topic

1.4 Novelty and significance of study

The novelty of this study lies in the development of a miniaturized 3D perfused microfluidic platform where the 3D fibrous scaffold is embedded within a stereolithography-printed microfluidic chip. The presence of fibrous scaffold mimics the cells ECM native environment and the local perfusion is favorable as it provides constant exchange of fluid and metabolites.

The incorporation of ECM-mimicking fibrous scaffold on a micro-perfused platform was hypothesized to better mimic the local *in vivo* cellular condition since there is both ECM fibrous structure and the presence of local perfusion. The resultant platform not only facilitates multiplexing for drug screening due to its miniaturized format; the device is better in maintaining cellular functions since it provides a more physiologically relevant micro-environment. [34].

This thesis sets out to validate the proposed idea using the current micro/nano technologies such as Electrospinning and 3D printing. A newly developed Electrospinning auxiliary jig from this study was capable of collecting both 3D fibrous aligned and random scaffold. Stereolithography was selected to build the micro-perfused platform where the 3D fibrous scaffold was embedded. This is the first instance where a miniaturized 3D fibrous scaffold was embedded on a Stereolithography-printed micro-perfused platform, presenting a highly relevant cell culturing platform. The device was further validated on the cell viability and functionality of Huh7.5 hepatocellular carcinoma cell line and presented in this study.

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Chapter 2

Literature Review

Currently the FDA drug approval process is considerably tedious and expensive with an average drug expenditure projected at \$1 billion in excess. To make matter worse, pharmaceutical companies faces huge risk when drug development failed at the later stage resulting in loss of lives and investment. Henceforth, there is an imperative need to develop a physiologically relevant in vitro model that would preserve the native functions of cells so as to better predict drug responses on human tissues. In this section, a review on the need of 3D microfluidic perfused culture will be previewed along with the current micro/-nano technology for the fabrication of the proposed device.

2.1 Introduction

Most cells are organized into three-dimensional (3D) format where the cell interactions with its surroundings are known to be critical factors in morphogenesis, proliferation and retaining its phenotypic functions [1]. However, the conventional 2D culture monolayer system often lacks the 3D cell interactions and is not an accurate reflection of the actual *in vivo* model. Many reported studies investigated the development of scaffolding materials which serve as an equivalent to the extracellular matrix (ECM) *in vivo*. The ECM matrix play an important role in providing structural support to the cells and regulating key cellular functions [2]. However, these scaffolds reported were either of 2D structures or 3D culture that lack the presence of ECM fibrous nature [3].

In the recent years, 3D cell cultures that adopted the use of hydrogel on a microfluidic platform were claimed to provide three-dimensionality for cells. However, these cell-laden hydrogel possess several limitations. The natural hydrogels often varies in their properties and composition where the transport of nutrients and metabolites can affect the viability of cells [4]. Currently, most tissue construct do not contain functional vasculature upon implantation and rely on either implanted blood vessels or *de novo* angiogenesis for delivering critical nutrients [5]. Since oxygen has low solubility and diffuses through length not greater than 200 μm , oxygen transportation is often the limiting factor for developing a functional 3D tissue construct [5].

Figure 2.1 presents a schematic explanation on limitation of nutrients diffusion for 2D versus 3D tissue construct [6]. Tissue construct of 2D monolayer platform rely on superficial diffusional transport mechanism which tend to fail to deliver medium to support three-dimensional tissues. As a result, nutrients and gaseous exchange are depleted over the distance where cells that are situated further cannot remain viable.

Figure 2.1 Schematic illustration of 3D necrotic tissue due to diffusion limitation versus healthy 2D construct

In order to address the issue on tissue diffusional limitation, bioreactors or perfused platforms have been developed to provide necessary nutrients to 3D tissue construct [7-11]. In general, the bioreactor or perfused platform should promote cell viability and maintain the phenotypic functions of cells. Numerous novel development has been made for bioreactors where the operational condition such as pH, temperature, and perfusion can be modified to suit various tissue needs [11, 12].

In the following section, an overview of the methodology for fabricating microfluidic perfused system will be previewed, along with its pros and cons. An emphasis on the different fabrication method of micro-tissue will also be discussed since the main focus of the thesis lies in the development of 3D micro-tissue on a 3D printed perfused platform.

2.2 Microfluidic cell culture system

Microfluidic culture system has become a valuable screening tool for biomedical research since it facilitates the analysis of cell-to-cell and cell-to-substrate dynamic responses [12]. Although 2D culturing technique is still a common laboratory practice, 3D cell culturing is rapidly gaining popularity due to greater awareness of the requirement for mimicking the cellular micro-environment [13].

Much effort has been focused on mimicking 3D cellular micro-environments since it better represent the *in vivo* physiological function which can be translated to better prediction of drug responses. Defined regulation of the spatial and temporal regulatory cues were found to be essential for the development of tissue construct. Cells grown on such physiologically-mimicking platform were found to have better cells proliferation and differentiation [14, 15]. Figure 2.2 provides an example of how perfusion could be adapted on a microfluidic platform to mimic the physiological condition of highly perfused organs, e.g., liver and kidney [16, 17].

Figure 2.2 Schematic of highly perfused organs on chip. (a) Example of microfluidic liver sinusoid where the microfluidic barrier mimics the highly permeable endothelial membrane [16]. (b) Example of a renal tubular epithelial cells platform that is exposed to a gradient of shear stress, hormonal stimulation and osmotic gradient [17].

While it is evident that cells cultured in physiologically-mimicking environment exhibit better hierarchical cellular organization and function [13] Widespread attention has been focused on the use of hydrogels such as, Matrigel, collagen, gelatin or gel methacrylate [13] to provide the three-dimensionality and compatibility to the natural ECM [6]. Take for example, mammary epithelial cells cultured in type I collagen gel exhibited milk protein secretion [18] and human hepatoma cell line HepG2 seeded on hydrogel form multiple polarized spheroids with increased gene expression of albumin and urea. Henceforth, it is of great interest to analyze the effect of perfusion on highly resolved 3D nanofibrous structure. Since these ECM structures were found to better represent the physiological environment of native ECM for cells study [19-21]

The basic 2D cell culture system such as tissue culture Polystyrene plates (TCPS) and microtiter plates remained as the common cell culture technique [13]. However, 3D cell culture paradigm is rapidly gaining acceptance due to the ability to create novel materials and intricate geometries that matches the cellular level. In the following section, microfluidic applications used for the culturing of cells on a perfused microfluidic chip is presented.

2.2.1 Examples of microfluidic cell cultures

A research group from Massachusetts Institute of Technology (MIT) has recently reported a perfusable 3D hepatic organoids system using both primary human hepatocytes and induced Pluripotent Stem cells-derived i-hepatocytes [22]. Hepatocytes (e.g. primary human hepatocytes or iPSc-derived hepatocytes) and fibroblasts are first centrifuged in the micro-wells and allowed to aggregate for 1 day. The cell aggregates are then encapsulated in PEG hydrogel to prevent uncontrolled growth and aggregation.

These aggregates when encapsulated have shown long-term preservation of the hepatic functions. Following which the micro-tissues are introduced into the PDMS microfluidic chip and entrapped by the C-trap architecture followed by media perfusion. Encapsulated cells exhibited viability on live dead staining. The reported platform yielded microtissues that gained stable albumin production on-chip and inducible CYP activity. Such approach has created opportunity for patient-specific drug screening applications [22].

Figure 2.3 Schematic of a perfusable 3D human liver platform. (a) Hepatocytes (e.g. primary human hepatocytes or iPSc-derived hepatocytes) and fibroblasts were centrifuged and allowed to aggregate for 1 day on microwell. (b) Encapsulated aggregates were seeded on microfluidic platform followed by perfusion. (c) i-hepatocytes demonstrating cell viability by calcein AM (green; live) and propidium iodide (red; dead). Scale bar represent 100 μm .

A recent demonstration by Hanry Yu's group on the development of a perfusion incubator liver chip is shown in Figure 2.3. The perfusion-incubator-liver-chip (PIC) is a self-sustaining chip with the integration of temperature, pH and bubble control. This system was reported to provide a contamination and clog-free environment for the maintenance of rat hepatocytes viability and metabolic activities for more than 3 weeks [23].

Figure 2.4 Schematic of the perfusion-incubator-liver-chip (a) 3D view of PIC. (b) Cross-sectional view of PIC

The ultrathin Parylene C porous membrane was used to constrain the hepatocyte spheroid and prevent excessive shear while providing good mass transfer. The integrated heater was connected to a temperature controller and thermometer where the feedback loop provided a continuous calibration of the device at 37°C. A pressurized carbon dioxide gas was drive across the media where pH of the culture was kept constant.

This multi-feature perfusion platform avoided the need of an incubator and it was kept in a biosafety cabinet to avoid contamination. The primary rat hepatocytes were found to be viable on the PIC device over 3 weeks along with significant improved or constant amount of urea, albumin and CYP450 enzyme activities [23].

Since the presence of a fibrous substrate on microfluidic cell culture is highly desirable for its bio-mimicking nature, Vanfleteren et al [24] reported a 2D fibrous membrane embedded on a biodegradable poly (polyol sebacate) substrate. The bio-mimicking nature of this reported microfluidic system was attributed to an embedded random poly L-lactic acid (PLLA) membrane fabricated using conventional electrospinning. The microfluidic substrate poly (polyol sebacate) was prepared via microwave polymerization for its biodegradability. The PLLA membrane was laser cut and positioned between the microfluidic channel during assembly and bonding process. The patency of the microfluidic platform was assessed by injecting ink into the channel where pressure drop was being measured, Figure 2.4 presents the assembled 3D biodegradable microfluidic platform.

Figure 2.5 Three-dimensional biodegradable microfluidic platform

Although the patency test has been performed on the microfluidic device, cell culture studies were lacking in this experimental setup. Firstly, the design does not support homogeneous cell seeding procedures and in addition should cells reside on the fibrous membrane the shear stress experienced by the cells will not be shielded and could be detrimental to its development. Last and most importantly, the fibrous membrane only provides a 2D micro-environment to the cells seeded on top of the membrane. From what has been discussed above, it is evident that 2D cultures are inadequate to represent *in vivo* conditions.

2.2.2 *Pros and cons of microfluidic cell culture system*

The microfluidic technology has enabled precise control of the spatial temporal cellular micro-environments by mimicking the complex biological functions of cell interactions. Microfluidic cell culture is a promising alternative to the conventional cell culturing method as it provides a well-defined and controlled micro-environment as well as enabling high throughput assays to be carried out. A variety of microfluidic devices has been developed for cell culturing studies as shown earlier in this chapter [25, 26].

The main benefit of microfluidic culturing system lies in the ability to handle small volume of fluids and reagents [3], the perfusion culture in specific allows precise distribution and removal of metabolites in a controlled micro-environment [27]. Most of the microfluidic cell culture reported were either based on the culturing of 2D monolayer cells or evolved around the use of hydrogel to provide the third dimensionality to the cells [28-30]. It has been reported widely that the presence of a 3D matrix supports many biologically relevant functions which has not been observed in any 2D culture. Henceforth, there is an increasing momentum in reports demonstrating the benefits of 3D cultures.

Among the many reported articles on 3D microfluidic cell culture, the culturing of cells in hydrogel have been demonstrated to be beneficial in retaining cell's native tissue-specific functions. The combination of hydrogel with microfluidics offers a variety of advantages which include [28]: (1) establishment of chemical gradients to recreate the dynamic 3D micro-environment; (2) provide suitable microscale dimensions that are equivalent to *in vivo* micro-environment; (3) create the appropriate matrix bio-interfaces along with temporal and spatial cellular control.

Despite the numerous benefits, a number of disadvantages using hydrogels microfluidic cell culture application still exist. The natural hydrogels come in differing properties and composition where the transport of nutrients and oxygen across it can affect the viability of cells cultured in them [13]. Due to the hydrophilic nature of hydrogel, the structures are easily disintegrated. The presence of bubbles and the introduction of cell-laden hydrogel in microfluidic channel prior to polymerization is also challenging.

While most of the hydrogel microfluidic platform claims to offer a 3D environment for cells, the presence of bio-mimicking fibrous extracellular matrix is missing. The native extracellular matrix (ECM), which is fibrous in nature, plays an essential role in providing physical cues to the cells and regulating key cellular functions [19]. Therefore, in this study, it is our aim to develop fibrous scaffold in the perfused microfluidic platform for further cell culturing studies. The embedded 3D fibrous scaffold coupled with well-defined microfluidic perfusion provides a better physiological micro-environment representation to study the dynamics of 3D cell-material interaction as well as to develop functional micro-tissues that could be used for drug screening [2, 20]. The synergistic effect of the fibrous scaffold on a perfused microfluidic platform is therefore beneficial to highly perfused organs such as liver or kidney where such a system could be used to extract meaningful data for cell based studies [5, 11, 31].

2.3 Development of 3D micro tissue

Over the past decade, the notion of tissue engineering of well-defined 3D tissues has received much attention for recapitulation of tissues and also pushed the boundaries for development of *in vitro* devices that retains the *in vivo* like functions of cells. Such models or platforms facilitate the extraction of more accurate and meaningful data during cell-assay studies. 3D micro tissues find application not only as functional biological tissues for grafting and other assay application but also as tumor models for the study of cancer biology as well as preclinical evaluation tools for drugs [1]. Figure 2.5 presents a collective summary of the different fabrication methodology of micro tissues.

Figure 2.6 Schematic of various 3D micro tissue formation method. (A) Hanging drop method [32]. (B) Microwell array [33]. (C) Formation of spheroids on a rotational flow microfluidic device [34]. (D) Cell-containing hydrogel microfiber generated by microfluidic coaxial cylinder system [35]

2.3.1 *Hanging drop method*

The hanging drop method is the most conventional approach to forming spheroids. In this method as shown in Figure 2.5A, a cell suspension is prepared at a suitable concentration and drops of suspension is deposited onto the well plate. The cell suspension then quickly forms a hanging drop within hours. The cells will subsequently agglomerate and form single spheroids within a day. The size of the spheroids can be tuned by changing the cell suspension concentration as well as the volume of the droplet [32].

Several groups have reported the use of hanging drop method such as those for the fabrication of 3D dermal spheres, the resulting 3D spheres were found to be similar to the dermal papilla morphologically [36]. Spheroids formed from tumor cells were also reported widely since the morphology of the cancer cells are better represented from the hanging drop method. Anticancer drug tested on such 3D model were found to be more effective than the common 2D cultures [37, 38]. However, the use of method is difficult to produce uniform spheroids of the preferred volume. In addition, this traditional method is not considered as a high-throughput system, the procedure is often considered tedious [32].

2.3.2 *Microwell array*

Cylindrical and concave microwell arrays fabricated by various micro technologies are considered an appealing tool for reproducing spheroids of identical size and shape [12]. A range of fabrication method and material has been used and were shown to be beneficial in this 3D format when compared to its 2D counterpart. Take for example, Fukuda et al [33]. created a patterned co-culture system with cylindrical microwell using chitosan hydrogels. As demonstrated in Figure 2.5B, the chitosan solution was coated on the glass and a PDMS mold was positioned on top of it. The chitosan was then polymerized by the use of UV light. HepG2 were seeded on the microwell and formed cell aggregates while NIH-3T3 cells were found to adhere on the chitosan substrate. The arrangement of this microwell array were found to be beneficial for the study of hepatotypic cell-cell interactions.

2.3.3 *Spheroid formation using rotational flow microfluidic chip*

Ota et al [34] has reported the formation of heterospheroids using a rotational flow microfluidic chip. In this method as shown in Figure 2.5C, a peristaltic pump was used to drive the flow of spheroids in a micro chamber where the size of spheroids can be controlled by changing the cell concentration and flow rate. The collected hepatocytes were coated with 200 nm of collagen and formed heterospheroids with endothelial cells within 2 mins. The major advantage of using the microfluidic setup is the presence of fluid flow that mimic the *in vivo* condition.

2.3.4 *Cell-laden hydrogel microfiber fabricated by microfluidic chip*

With the recent development of microfluidic spinning technology, Onoe et al [35] has demonstrated the fabrication of a meter-long hydrogel microfibers to be stacked into 3D tissue construct. The hydrogel fiber was collected using a double-coaxial, laminar flow microfluidic device. In this setup, the cell-laden hydrogel was collected using three different solutions: 1) the core stream which contain an ECM pre-gel solution of cells; 2) the shell stream containing Na-alginate in saline; and 3) the sheath stream containing a solution of CaCl_2 and sucrose. Various types of cell including fibroblasts, myocytes, endothelial, nerve and epithelial cells were encapsulated in the hydrogel microfiber. Cellular morphology and functions were found to be retained upon removal of hydrogel sheath. While encapsulation of cells in hydrogel enables the construction of various tissue shape, the exchange of nutrients and oxygen in the 3D construct remained to be challenging.

2.4 3D printing technologies in microfluidic development

Microfluidics is a thriving field that enable an extensive range of screening and biological applications such as high-throughput drug testing, cancer screening and point-of-care diagnostic [39]. However, the fabrication of microfluidic devices is often tedious, complex and requires sophisticated cleanroom facilities [13]. Three-dimensional (3D) printing on the other hand presents a favorable alternative to conventional microfluidics fabrication techniques such as lithography and PDMS-glass bonding [10, 39]. The 3d printing process is an ideal tool that enable rapid design iterations in the initial design stage. Significant cost reduction in infrastructure, equipment and maintenance is also made possible. In this section, two additive manufacturing techniques, namely Fused Deposition Modeling (FDM) and Stereolithography (SLA) was selected for the use of microfluidic development.

2.4.1 Fused Deposition Modeling (FDM) and its application in microfluidics

The FDM method evolves around the use of thermoplastic filament to print layers of materials and form 3D structures, as shown in Figure 2.6A. The polymeric filament is first heated at the nozzle and when it reaches a semi-liquid state, material will be collected on the platform or previously printed layers [40]. The main processing parameters that affects the scaffold mechanical properties lies in the layer thickness, width and orientation. Inter-layer distortion has been reported to be the main reason of mechanical weakness [41]. The main benefit of FDM process is the low cost, high speed as well as process simplicity. However, the poor mechanical properties, layer-by-layer sealing and surface finishing are the limiting factors to be used in microfluidic chip [40].

L.Cronin et al [42] reported a reactionware printed by FDM for chemical synthesis and analysis as shown in Figure 2.6B. In this study, a reactionware was printed using a cheap FDM process where the setup enables reaction to be monitored *in situ* [42]. Different reactionware architecture and chemistry could be used for screening of their efficacy and provide the necessary feedback.

Figure 2.7 Schematics of Fused Deposition Modeling (FDM) process and its application. (A) Example of the FDM printing process. (B) FDM-printed reactionware. (C) Schematic of the multipurpose reactionware used for synthesis of compounds.

2.4.2 Stereolithography and its application in microfluidics

Stereolithography is one of the earliest additive manufacturing technique developed in late 1980s [43]. This technique uses light energy (UV light or electron beam) to cure the liquid resin as shown in Figure 2.7A. The liquid resin is light sensitive and instantly convert to a layer of polymer upon UV activation. After polymerization, a layer of pattern resides on the moving platform which enable to hold the subsequent layer hence building into 3D structures. The uncured resin can be removed upon printing and a post-curing process such as photo-curing can be used to retain the mechanical properties. While SLA is the only additive manufacturing technique that prints high-quality and highly resolved structures up to 50 μm [40]. The process is relatively slow, expensive and the available material is currently limited. However, due to the high-quality surface finishing of the printed part, many reports were based on SLA-printed microfluidic chip [39, 44, 45].

Figure 2.8 Schematics of Stereolithography process and its application. (A) Example of Stereolithography printing process. (B) Exploded view of SLA-printed microfluidic chip. (C) Microfluidic device setup with luer interface.

Louis J.Y. Ong et al has recently reported a SLA-printed perfused microfluidic device for the culturing of HepG2 cells, as presented in Figure 2.7B & C [44]. In this setup, the top and bottom layer of the microfluidic chip was 3d printed using Polycarbonate and post-cured for 2 hours at 60°C. The device is subsequently assembled with a 170 μm PDMS membrane for cell entrapment and glass slide as viewing window, the whole assembly is secured with M5 stainless steel screws.

This device has demonstrated the capability of immobilizing and maintaining the viability and functionality of the carcinoma liver cells. At the same time, the pump-free perfusion system relies on gravity-driven flow to perfuse the cancer cells. Such a setup reduces the handling complexity as well as power consumption of the device.

While the culturing of cells on a 3D format along with perfusion presents an exciting opportunity as described in the above few instances, the absence of fibrous micro-environment fails to capture the essence of *in vivo* culture. Hence in the subsequent section, techniques on the collection of fibrous scaffold will be reviewed and this is followed by a summary on the literature review presented.

2.5 Scaffold fabrication process: Electrospinning

Biomimetic scaffolds with fibrillary and porous nano-features are highly desirable since it promotes essential cellular processes [46]. Several nanofabrication techniques initially developed for various purposes have been adopted for scaffold fabrication process [20]. Of which Electrospinning is one of the commonly used technique for the fabrication of 2D scaffolds comprising of interwoven fibers [19, 47]. The nanofibers fabrication method uses an electric potential to control the deposition of fibers on a targeted collector [48]. The fibers formed are usually continuous in either a random orientation or well-aligned by using a stationary or rotating collector respectively.

Figure 2.9 Schematics of various electrospinning setup. (a) Static counter-electrode [48]. (b) Rotating drum [49]. (c) Water bath counter-electrode for collection of thick 3D random scaffold [50]. (d) Parallel plate collector for the collection of aligned scaffold [48].

Unconventional electrospinning techniques such as the water bath collector enable the formation of thick 3D random oriented scaffold [50]. These scaffolds are made up of continuous fibers, with high porosity and spatial interconnectivity which address the limitation of poor cells and reagent infiltration on 2D substrates [46, 50]. In the following chapters, the electrospinning auxiliary jig developed in this study will be presented. The auxiliary jig was designed to have the versatility to collect both 3D aligned and random scaffold which will be embedded on a micro-perfused platform.

2.6 Summary

In summary, a review on the current 3D cell culture system on microfluidic platform has been presented. The advantages and disadvantages has been discussed and found that such systems were mainly of hydrogel or 2D format that lack the fibrous ECM nature. The electrospinning technique has been reviewed where modified technique is much preferred since it could recapitulate the 3D fibrillary nature of ECM structure. The 3D printing technologies has been discussed and Stereolithography was found to give a higher resolution. Henceforth, in this study an electrospun 3D fibrous scaffold will be embedded on a Stereolithography-printed micro-perfused platform to better mimic the cellular micro-environment for future cell culture studies.

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Chapter 3

Experimental Methodology

The experimental methodology chapter presents the design, fabrication and characterization techniques used to address the objectives of this study. The design of electrospinning auxiliary jig and the one-step stereolithography process for the fabrication of micro-perfused cell culturing platform has been discussed. A series of physical characterization has been performed on the 3D porous scaffold to determine its properties which were later used in Computational Fluid Dynamic (CFD) simulation for the optimization of perfusion flow culture. Cell culture studies were performed on the SLA-printed perfused microfluidic platform where a series of cell viability, proliferation studies and gene expression of Huh7.5 model cells were conducted. The principles underlying the process and characterization method were discussed and the analytical parameters of the methodologies were described in detail.

3.1 Materials

3.1.1 Polycaprolactone (PCL) solution for electrospinning of 3D aligned and random scaffold for human aorta smooth muscle cells (hSMCs) study

Polycaprolactone material was used to fabricate scaffold with 3D aligned and random orientation in this study. Polycaprolactone (PCL) pellets (Capa™ 6800 with average molecular weight of 80,000 amu) purchased from Perstorp was dissolved in 6.5:3.5 ratio of dichloromethane (DCM) and dimethylformamide (DMF), magnetic stirred for 24 hours to obtain a 13 wt% polymer solution. Polymer solution was fed at a rate of 0.5 to 1.0 ml/h to a flat tip stainless steel spinneret which is connected to a high-voltage power supply. Electrospinning was carried out at ambient temperature (20 – 24°C) and at a range of accelerating voltage from 16 to 21kV, while the collector remained earthed. The PCL polymer solution was pumped to a 21-gauge needle with a collector distance optimized between 4 to 20 mm. The rotation speed for collection of 3D aligned fibers was optimized between 500 to 800 rpm, while the collection of 3D random scaffold was performed without any form of rotation. The bath solution used in this study has been optimized based on an investigation of wettability between the polymer solution and the bath solution [1]. The concentration of 50:50 isopropyl alcohol (IPA) and deionized water was found to promote the infiltration of fibers into the collector bath making the scaffold more porous as compared to the conventional electrospinning process. All scaffolds were collected after 30 mins of spinning with 5 mins of cleaning frequency and placed in freezer for approximately 24 h prior to freeze drying.

3.1.2 Poly(lactic-co-glycolic acid) (PLGA) solution for electrospinning of micro 3D random scaffold

Poly(lactic-co-glycolic acid) (PLGA) was used to fabricate micro 3D random scaffold which was later embedded in the micro perfused setup. Poly(lactic-co-glycolic acid) (PLGA) 80:20 (Resomer® LG 824S) was purchased from Evonik and dissolved in 7:3 ratio of Chloroform and dimethylformamide (DMF) magnetic stirred for 24 hours to obtain a 8

wt% polymer solution. The electrospun polymer solution was fed at a rate of 0.5 to 0.8 ml/h to a flat tip stainless steel spinneret connected to a high-voltage power supply. Electrospinning was carried out at ambient temperature (20 – 24 °C) and a range of accelerating voltage from 16 to 18 kV was used with the collector remained earthed. The PLGA polymer solution was pumped to a 21-gauge needle with a collector distance optimized between 4 to 10mm. The 3D random PLGA fibrous scaffold were used for cell viability studies on the miniaturized perfused platform. The bath solution optimized by the Wilhelmy plate method were found to be 70:30 IPA and deionized water for the collection of porous structure. All scaffolds were collected after 30 mins of spinning with 5 mins of cleaning frequency and placed in freezer for approximately 24 h prior to freeze drying.

3.1.3 *Polydimethylsiloxane (PDMS)*

PDMS prepolymer (Sylgard 184, Dow Corning, MI, USA) was mixed with curing agent at ratio of 10:1 by weight. The PDMS solution was degassed and casted on a Teflon frame, cured at 80°C for 3 hours. The final PDMS sheet was removed and punched to a circular diameter of 10mm to be embedded on the microfluidic perfused platform. Prior to using the PDMS sheet, measurement was first taken and found to be in the range of 0.22 to 0.25 μm .

3.1.4 *NextDent Ortho Clear resin for Stereolithography printing*

NextDent Ortho Clear a biocompatible Class IIa material was used for the Stereolithography printing of perfused microfluidic platform. The final optimized processing condition for the resin was having a light intensity of 16 mW/cm² and an exposure time of 0.45s.

3.2 Electrospinning Process

3.2.1 Fundamental of Electrospinning

Biomimetic scaffolds with fibrillary and porous nano-features are highly desirable since it promote essential cellular processes [2]. Several nanofabrication techniques originally developed for various purposes have been adopted for scaffold fabrication process. Electrospinning [3, 4] and molecular self-assembly [5] are commonly used for the fabrication of 3D scaffolds comprising of woven fibers [2]. Electrospinning is a facile nanofibers fabrication method that uses an electric potential to control the deposition of fibers on a targeted collector [6]. The fibers formed are usually continuous, either in random or aligned format by using a stationary or rotating collector respectively, Figure 3.1a and 3.1b [7].

Figure 3.1 Schematics of various Electrospinning Setup. A. Static counter-electrode demonstrating the formation of taylor cone and collection of randomly orientated fibers [6]. B. Solid rotating drum for the collection of aligned fibers [8]. C. Water bath counter-electrode for collection of random 3D fibers [9]. Parallel plate collector for the collection of aligned fibers [6].

Unconventional electrospinning techniques such as the water bath collector enable the formation of thick highly porous 3D scaffold as shown in Figure 3.1c [9]. These scaffolds are made up of continuous fibers, with high porosity and spatial interconnectivity which address the limitation of poor cells and reagent infiltration on 2D substrates [2, 9]. In general, at some critical electrical field, electrospun jet overcome the surface tension of the Taylor cone in order to form jets of polymer solution. As the polymer jet thin towards the collector, surface tension becomes relevant to fibers quality. Henceforth, ionic surfactant can be added to the water bath to increase the conductivity while reducing the surface tension of the water bath. This will ensure the formation of homogeneous fibers formed in the water bath with good porosity [9].

The collection of fibers on a pair of split electrodes has been widely reported as shown in Figure 3.1d [6, 10]. Under the action of opposite electrostatic force (e.g. Strong coulombic interactions between the positive charges on fibres and negative charges on the 2 grounded electrodes), the charged fibers stretched and align perpendicular to each edge of the gap [10]. In addition, further fiber alignment within the gap is attributed from the electrostatic repulsions between deposited nanofibers.

The collection of nanofibers in this format makes the transfer of aligned fibers onto other solid substrate easier. The stacking of such fibers films promised the formation of complex scaffold architecture. In addition, single fibers could be transfer for the fabrication of single fiber based devices [6].

3.2.2 Development of auxiliary jig for Electrospinning of 3D aligned & random scaffold

All electrospun scaffolds were prepared on Nanofiber Electrospinning System, Nanon-01A along with the newly designed auxiliary setup, as shown in Fig. 3.2. The electrospinning auxiliary setup designed in this study aims to collect 3D fibrous aligned and random scaffold. The white Teflon bath was used to prevent fibers from attaching to the edges of the bath, while the rotating mechanism as shown in Figure 3.2b is embedded within the bath and attached to the rotating shaft of the electrospinning machine.

The auxiliary setup is capable of collecting both 3D aligned and random scaffold. Well-aligned and porous scaffold were collected on a tubular format where fibres are aligned circumferentially and at the same time with sufficient substrate thickness. Random 3D fibrous scaffold can also be fabricated on the auxiliary setup without attaching the rotating mandrel in the bath. The bath solution has to be optimized using the Wilhelmy plate method depending on the polymeric material used.

Figure 3.2 Illustration of the rotating bath electrospinning setup. (A) Schematics of rotating bath electrospinning setup. (B) Top view of auxiliary jig with tunable stainless steel electrode.

3.2.3 Optimization of wet electrospinning process through Wilhelmy plate method

Figure 3.3 Schematics of Wilhelmy plate method

The surface tension of the electrospinning solution and bath solution was measured using the Kruss K100 tensiometer via the Wilhelmy plate method. The Wilhelmy method describes the contact angle between a solid and a liquid as shown in Equation (1). The wetting force F , which is also known as the capillary force exerted by the liquid on the solid sample, was recorded by the tensiometer. In order to determine surface tension/energy of the liquid, a Platinum plate was used. Since Platinum has high surface energy and is assumed to perfectly wet most of liquids, the contact angle between the Platinum probe and the test liquid sample is zero, hence $\cos \theta$ is 1. The wetted length L of the plate is equal to its perimeter. Therefore, with the measured wetting force F , the surface tension of test liquid can be calculated according to the Equation (1)

$$\sigma = \frac{F}{L \cdot \cos \theta} \quad [1]$$

3.3 Stereolithography Process

3.3.1 Fundamental of Stereolithography process

The Stereolithography process is a form of 3D printing where 3D objects can be replicated by using a focused light source. The Asiga Pico2 HD 27 was used in this study for the fabrication of micro-perfused culture. NextDent Ortho clear resin has been used in this study as the photocurable resin. Briefly, the micro-perfused culture was replicated layer-by-layer as shown in Figure 3.4, with the use of selective light exposure to polymerize the liquid monomer on an Asiga build tray (shown in blue). Each of these layer is projected as an image obtained from sectioning the 3D design on the Composer software. The process starts with sweeping a thin layer of resin on the platform as shown in Figure 3.4 a and b. This is followed by selective light exposure of the design (Figure 3.4c) to photo-polymerize the thin layer of resin hence creating the 3D construct layer-by-layer. The photo-polymerized layer is subsequently separated from the build tray (Figure 3.4d) in preparation for the printing of next layer.

Figure 3.4 Schematic illustration of Stereolithography process. (A) Preparation of printing process. (B) Imprinting of a thin layer of resin on platform. (C) Selective light exposure of design. (D) Separation of design from build tray

3.3.2 Fabrication of microfluidic perfused platform using one-step Stereolithography process

The design of Stereolithography-printed perfused microfluidic platform is shown in Figure 3.5. The SLA-printed perfused microfluidic platform comprises of 3 distinct layers where each layer has its own functionality. The whole device was fabricated via Stereolithography process where each layer is printed on top of another (e.g. layer 1 followed by layer 2 then layer 3). To further illustrate, the process starts off with the printing of 1st layer used for visualizing of the perfusion activities. Upon completion of first layer, a piece of PDMS is fitted into the gap where it provides a seal for the cell culturing chamber.

Figure 3.5 Design of Stereolithography-printed perfused microfluidic platform

The second layer consisting of the cell culturing chamber as well as the micro-channels for delivery of cell culture medium will be printed on top of the first layer. Upon completion of layer 2, the layer was rinsed with isopropyl alcohol (IPA) to remove any residual resin in the cell culturing chamber. Following which, a miniaturized piece of PLGA 3D fibrous

scaffold was introduced to the micro-chamber and sealed with another piece of PDMS, see Figure 3.6 for the cross-sectional view of the micro perfused setup. The final layer of the SLA-printed platform ends with the printing of user interface where the ferrules are printed for media exchange.

Figure 3.6 Cross-sectional view of Stereolithography-printed perfused microfluidic platform

The detailed dimensioning of the device and each individual layer were provided in Appendix A.

3.4 Physical Characterization

3.4.1 Scanning Electron Microscopy (SEM)

Figure 3.7 Schematics of Scanning Electron Microscopy (SEM)

The Scanning Electron Microscopy (SEM) was used to visualize electrospun scaffold. Figure 3.6 presents the schematics of a scanning electron microscopy (SEM). The working principles of the SEM starts off with the specimen being probed by a beam of electrons across the sample. The electrons are emitted from the electron gun by thermionic heating and condensed into a narrow electron beam.

The three most common types of electron sources include: tungsten filament, solid state crystal (LaB_6) and field emission gun (FEG) [11]. The tungsten filament is the most basic type of filament while solid state crystal possesses a longer lifespan than tungsten. The field emission gun is also a tungsten wire but shaped into a sharp tip. The FEG generate electrons by placing the filament in a huge electrical potential gradient hence obviating

issues found in thermionic sources such as low brightness, evaporation of cathode material or thermal drifting during operation.

A series of condenser lens will be used to focus the electron beam and the scanning coil will be used to deflect the focused beam in X and Y axes to allow the beam to scan in a raster fashion.

Radiations from the specimen are detected, amplified and used for various analysis such as secondary electrons (for SEM images) or characteristics X-rays for elemental analysis. Secondary electrons are low energy electrons that are emitted at region near the sample surface hence generating surface topography. On the other hand, backscattered electrons (BSE) are electrons reflected from a deeper region and are closely correlated to the atomic number of the sample. Therefore, BSE images are used to derive compositional information of samples.

Both the Jeol 6360 thermionic scanning electron microscopy (SEM) and Jeol 6340F field emission scanning electron microscopy (FE-SEM) was used for both microscopic and close-up images respectively. The samples were imaged at accelerating voltage of 5kV and a working distance between 10-15 mm. All samples were coated with gold prior to imaging to increase the sample conductivity. Scaffolds were immersed in liquid nitrogen for 5 mins, sectioned across the thickness prior to imaging the cross-sectional view.

3.4.2 2D Fast Fourier Transform (FFT) alignment

2D Fast Fourier transform (FFT) was performed to determine the fibre alignment in electrospun scaffold. The SEM image was uploaded to ImageJ[®] software where the image is processed by 2D FFT [12]. The resulting frequency plot would contain a mass of white pixels depending on the nature of the image processed. Following which a rotation of 90° on the frequency plot was performed to compensate for the intrinsic rotation of the data induced by FFT analysis. The frequency plot is then analyzed by a supported oval profile plugin where the intensities of the pixels are summed along the radius from e.g., 0 – 360° and normalized to a baseline value of 0. Peaks of nearly uniform height is a characteristic of scaffold containing random features, while a highly aligned fibre scaffold would produce prominent peak in the 2D FFT alignment plot.

3.4.3 Scaffold porosity & pore size measurement

The scaffold porosity was measured by liquid intrusion method [13, 14] where the weight of dry scaffold was first measured prior to immersion in 100% ethanol overnight. The scaffold was gently wiped to get rid of any excess ethanol on the surface and weighed again. Scaffold with ($n = 3$) was calculated using the following equations:

$$\text{Scaffold porosity} = \frac{V_{eth}}{(V_{pcl} + V_{eth})} \times 100\% \quad [2]$$

Where:

$$V_{eth} = \frac{(W_{wet} - W_{dry})}{\rho_{eth}} \quad V_{pcl} = \frac{W_{dry}}{\rho_{pcl}}$$

V_{eth} denotes the volume of ethanol entrapped in the scaffold; W_{dry} and W_{wet} denote the dry and wet weight of the scaffold respectively; ρ_{eth} denotes the density of ethanol (0.789 mg/mm³); ρ_{pcl} denotes the density of polycaprolactone (1.145 mg/mm³)

The average pore size of the scaffold was determined by measuring at least 50 pores per sample using the ImageJ[®] (NIH, Bethesda, Maryland, USA) software. The pore size is defined as the distance between fibres and expressed as mean $\pm$ standard deviation.

3.4.4 Contact angle measurement

The hydrophobicity of the 3D aligned and random scaffold was measured using the static water contact angle (WCA) technique on Dataphysics OCA system [15]. The contact angle is the angle formed at the intersection between solid-liquid and liquid-vapor interface. The wettability studies show the degree of wetting upon contact between a solid and liquid. A hydrophilic surface corresponds to a smaller contact angle ($<90^\circ$) while a hydrophobic surface corresponds to a larger contact angles ($>90^\circ$).

The measurement starts the use of a G25 gauge needle where the sessile droplet at a volume of 6.0 μl and dosing rate of 1.0 $\mu\text{l/s}$ was formed. One droplet of distilled water was dropped on each sample ($n = 3$) where the projected images of droplet settled on the fibrous scaffold showing no noticeable changes was analyzed. The SCA 20 software was used to profile the droplet and the contact angle measurement was read off.

3.4.5 Mechanical Properties

The Instron machine testes a series of mechanical properties such as tensile, fatigue and impact tests [16]. In this study, the tensile strength and Young's modulus of the sample was tested on the Instron machine. Aligned and random scaffolds were fabricated and cut according to ASTM D638 dimensions. Mechanical properties of aligned scaffolds were measured along the fibre direction ($//$). The cross-sectional area of the samples ($n = 5$) were measured using micrometer and loaded onto an Instron 5567 testing machine, where testing were conducted using 10N load and crosshead speed of 10 mm/min. The gauge length was determined as the distance between the grips. All testing were carried out at ambient temperature. Strain was defined as extension normalized to gauge length; stress was calculated as the load normalized to initial cross-sectional area. The modulus was computed as the slope of stress-strain curve.

3.5 Perfusion flow setup

3.5.1 Computational Fluid Dynamic (CFD) simulation

The computational fluid dynamic simulation Flow3D was used to predict the flow behavior of medium in the micro-perfused culture. In the simulation, effect of channel dimension and flowrate on fluid shear stress has been analyzed. Since liver hepatocytes is highly shear-sensitive, the simulation will provide insights on the acceptable limits for flowrate and channel width such that the overall shear stress of the device remained below 0.1Pa [17, 18].

The shear stress experienced by the wall is governed by a simple 2D Poiseuille flow system described in equation 3:

$$\tau = \frac{6\mu Q}{h^2 w} \quad [3]$$

where, μ defines viscosity of fluid, Q as flowrate, h as channel height and w as channel width.

The pressure loss related to the embedded fibrous scaffold has been considered through the Forchheimer saturated drag model. In Forchheimer's equation, pressure drop is defined as [19, 20]:

$$-\nabla p = (A + B|\mu_{bulk}|)\mu_{bulk} \quad (3)$$

where A and B are coefficients defined by experimental data, μ as the apparent velocity and ∇p is the pressure gradient in real space within the porous medium.

$$A = 180 / D_{fiber}^2 \quad B = 2.0 / D_{fiber}$$

The average fiber diameter of the scaffold used in the Flow3D simulation was measured on ImageJ and found to be $0.95 \pm 0.038 \mu\text{m}$.

The parameters used for the simulation has been summarized as below:

Overall simulation key settings:

1. Fluid used in simulation studies: Water
2. Cell size used in simulation studies: 0.0001 m
3. Forchheimer saturated drag was used to define the porous media in the chamber and has been described in results section

Effect of channel dimension (width):

- Three different channel dimension have been used in this study: 300 μm , 600 μm , 800 μm
- Volumetric flow rate: 18ml/hr
- Chamber porosity: 85%; Drag Coefficient A – $1.8\text{e}+11 \text{ 1/m}^2$; Drag Coefficient B – $1.8\text{e}+05 \text{ 1/m}$ (refer to chapter 5 for more explanation)

P.S: Any volumetric flow rate lower than 18 ml/hr would require long simulation hours and large computing space

Effect of volumetric flowrate:

- Three different volumetric flowrates have been used in this study: 360 ml/hr, 180 ml/hr, 0.18 ml/hr
- Channel width: 800 μm
- Chamber porosity: 85%; Drag Coefficient A – $1.8\text{e}+11 \text{ 1/m}^2$; Drag Coefficient B – $1.8\text{e}+05 \text{ 1/m}$ (refer to chapter 5 for more explanation)

P.S: Any volumetric flow rate lower than 18 ml/hr would require long simulation hours and large computing space

3.5.2 Description of perfusion setup

The Stereolithography-printed perfusion platform were placed in a six well-plate for perfusion culture studies as shown in Figure 3.8. The SLA-printed device were soaked in 70% Ethanol for 30 mins and vacuum for 5 mins to remove residual bubbles. The device was flushed with 10ml of PBS followed by 10ml of cell culture media. The whole device was soaked in a culture media overnight to ensure complete wetting of the embedded scaffold.

Huh7.5 human hepatocarcinoma cell line was injected at a density of 5×10^5 cells per well through the central port and allowed to be attached on the scaffold overnight. The cell culture medium was perfused at a controlled rate optimized by the Flow3D simulation (e.g. 0.12 ml/h) through a syringe that is connected to a syringe pump. The perfusate was collected from the other side of the well and prepared for Albumin ELISA assay.

Figure 3.8 Schematics of perfusion culture setup

3.6 Cell culture studies

3.6.1 Cell culture and seeding

Two types of cell culturing activities have been performed in this study. The cell viability test was performed on the Stereolithography printed perfused platform where Huh7.5 human hepatocarcinoma cell line was used as the model cells. Human aorta smooth muscle cells were used in this study to demonstrate the effect of 3D aligned fibres on the phenotypic maintenance due to topographical cues. The cell culturing and seeding protocol performed is reported as follows.

Huh7.5 human hepatocarcinoma cell line was purchased from Riken (VA, Japan). The Huh7.5 cells were maintained in Dulbecco's modified eagle medium (DMEM, Hyclone, GE Healthcare and Life Sciences) supplemented with 10% foetal bovine serum (Gibco, Life Technologies) and 1% penicillin/streptomycin (Gibco, Life Technologies). The seeding density of 5×10^5 cells per SLA-printed device or well were used for cell viability, proliferation and gene expression studies. The SLA-printed device was perfused at a rate of 0.12ml/h and the medium for static culture was refreshed every other day.

Human aorta smooth muscle cells (hSMCs) used in this study was obtained from Lonza Clonetics® and expanded up to passage 7 to 9 on gelatin coated culture flasks. Cells were maintained in SmBM basal medium along with supplemented growth factors kit from Lonza and 1% antibiotic-antimycotic (AA) at 37°C with 5% CO₂. For cell seeding, fibrous membrane upon freeze drying were cut to fit into 24 well plate, soaked in 70% ethanol for 1 hour, sterilized by UV irradiation for 1 hour on each side and rinsed in PBS three times. The membranes were incubated overnight with cell culture prior to cell seeding. The hSMCs were seeded onto the fibrous scaffolds at a density of 3×10^5 cells per well for cell morphology, proliferation and qualitative differentiation studies. The medium was refreshed every other day.

3.6.2 *Cell seeding efficiency*

The cell seeding efficiency test has been performed on the SLA-printed perfused microfluidic platform to determine the optimal cell density to be seeded for future cell culture studies. Three different cell densities, 5×10^5 , 5×10^6 and 1.5×10^7 were seeded into the microfluidic platform through the cell inlet port. Cells were allowed to be incubated for 16 hours prior to retrieving the embedded scaffold for WST-8 assay. The retrieved scaffold is incubated with 1 ml of medium with 1:10 WST-8 reagent to medium. The reaction was incubated at 37°C and 5% CO₂ for 2 h. After which, 100 µl aliquots of each well were transferred to a fresh 96 well plate where absorbance readings were taken at 450 nm on a microplate reader. Each experiment was performed in triplicate.

3.6.3 *Cell proliferation*

Cell proliferation was analyzed by WST-8 assay (Dojindo Molecular Laboratories Inc., Kumanoto, Japan). Briefly, on the designated time point the medium was aspirated, and the cell culture medium in each well is replaced with 1 ml of medium with 1:10 WST-8 reagent to medium ratio. The reaction was incubated at 37°C and 5% CO₂ for 2 h. After which, 100µl aliquots of each well were transferred to a fresh 96 well plate where absorbance readings were taken at 450 nm on a microplate reader. Each experiment were performed in triplicate.

3.6.4 *Cell viability analysis*

The cell viability test of Huh7.5 on the micro-perfused platform has been performed using the LIVE/DEAD cell viability assay (Life Technologies). Cells were incubated in 2 µM Calcein-AM and 4µM Ethidium homodimer (EthD-1) at the specified time point for 15 mins at 37°C and 5% CO₂. The cytoplasm of live cells was stained by Calcein-AM (green) while the nucleus of dead cells was stained by EthD-1 (red). The samples were imaged via confocal microscopy (Carl Zeiss LSM 700, AG, Oberkochen, Germany).

3.6.5 *Cell infiltration analysis*

The cell infiltration studies were performed to determine the depth of cell penetration on the perfused scaffold. Briefly, scaffolds were rinsed with PBS and fixed with 4% paraformaldehyde (PFA). The samples were subsequently soaked in 15% sucrose and transferred to a 30% sucrose solution for 2 hours each. The scaffolds were then embedded in OCT freezing medium overnight for sufficient infiltration and allowed it to freeze down to -20°C. Cryo-sectioning was performed where 30 µm thickness were collected on a glass slide. Immunofluorescence staining was performed where Nuclei were stained with DAPI (1:1000 dilution in 5% BSA, Life Technologies). The fluorescence images were visualized under a fluorescence microscope (CX 51; Olympus, Japan).

3.6.6 *F-actin staining for cytoskeleton structure*

The cytoskeleton of smooth muscle cells was studied after 1, 7 and 21 days of culture where the filamentous actin (F-actin) and cell nucleus were counter stained with rhodamine-phalloidin (1:200 dilution in 5% BSA, Life Technologies) and 4'-6-Diamidino-2-phenylindole (DAPI) (1:1000 dilution in 5% BSA, Life Technologies) respectively. Briefly, hSMCs were fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature and subsequently blocked with 5 % bovine serum albumin (BSA) in PBS before the subcellular components were immune-labeled. The fluorescence images were visualized under a fluorescence microscope (CX 51; Olympus, Japan).

3.6.7 Immunocytochemistry analysis

The expression of early and late markers of the hSMCs, namely α -smooth muscle actin (α -SMA) and heavy myosin chain (MHC) respectively, were evaluated using immunocytochemistry analysis. Cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, washed 3 times in PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. After three washes with PBS, the cells were blocked with 10% goat serum (normal, Dako, Denmark) for 1h. The samples were subsequently incubated with mouse monoclonal anti alpha smooth muscle actin (ab7817) (1:400, abcam) and rabbit polyclonal anti smooth muscle myosin heavy chain 11 (ab53219) (1:200, abcam) overnight at 4°C. Alexa Fluor secondary antibodies 488 goat anti-mouse IgG (1:200, Molecular probes, Invitrogen) and 568 goat anti-rabbit IgG (1:100, Molecular probes, Invitrogen) were used to counter stained its respective antibodies. Nuclei were stained with DAPI (1:1000 dilution in 5% BSA, Life Technologies). The fluorescence images were visualized under a fluorescence microscope (CX 51; Olympus, Japan).

3.6.8 Albumin quantification

Albumin secretion in hepatocyte culture medium was quantified using enzyme-linked immunosorbent assay, Albumin human Elisa kit (Abcam). The assay was performed as per manufacturer's instructions where the absorbance readings were taken at 450 nm on a microplate reader. Each experiment was performed in triplicate.

3.6.9 Quantitative real time polymerase chain reaction (qRT-PCR)

At specific timepoint scaffold were removed from the experiment where the total RNA was lysed using Trizol (Life Technologies). Chloroform was added to the cell lysates where the RNA was extracted in the aqueous phase. The precipitation of separated RNA was obtained through isopropyl alcohol and rinsed with 75% ethanol twice to remove impurities. The

final RNA pellet was put together in RNase free water where ND-1000 spectrophotometer (NanoDrop Technologies Wilmington, DE) was used to check for its quality and quantity.

First strand complement DNA (cDNA) was synthesized with iScript™ cDNA synthesis kit, where the qPCR was performed on CFX96 real time PCR detection system (Bio-rad Laboratories, Inc, USA). The reaction mix comprises of 10µl of SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 1µl of forward and reverse primers (5µM) respectively, 1µl of diluted cDNA and PCR grade water to make up a final volume of 20µl. The primers of interest were obtained from Integrated DNA Technologies (Coralville, IA) as shown in Table 3.1. GAPDH was used as the housekeeping gene and $\Delta\Delta CT$ method was used to analyze the relative gene expression levels of the target genes.

Table 3.1 List of primer sequences used in amplification

Gene	Forward primer (5'-3')	Reverse primer (3'-5')
Albumin	CTG CAC AGA ATC CTT GGT GA	CTC CTT ATC GTC AGC CTT GC
CYP3A4	ACC GTG ACC CAAAGT ACT GG	TTC AGG GGG ATC TGT GTT TC
CYP3A7	AAG TGG ACC CAG AAA CTG CA	GGC TCC ACT TAC GGT CTC AT
GAPDH	CAT GAG AAG TAT GAC AAC AGC CT	AGT CCT TCC ACG ATA CCA AAG T

3.7 Statistical analysis

Results are presented as mean $\pm$ standard deviation where statistical differences are determined by student's *t*-test and are considered statistically significant at $p \leq 0.05$ or $p \leq 0.01$.

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Chapter 4

An Electrospinning technique for the collection of 3D aligned & random scaffold

The conventional Electrospinning technique often deliver fibrous scaffold on a 2D format. However, cells sense, interact and respond dynamically within its micro-environment, hence there is an imperative need to develop scaffold on a 3D format. In this chapter, an electrospinning technique has been reviewed for the collection of 3D aligned and random fibrous scaffold. The Wilhelmy plate method has been adopted to optimize the electrospinning process and a series of physical characterization has been conducted on the scaffolds. The collected 3D aligned and random fibrous scaffolds were found to be architecturally porous which may better serve as a physiologically equivalent tissue construct.

4.1 Introduction

Electrospinning is a flexible technique that is capable of fabricating micro to nano-sized fibers [1, 2]. Such fibrous nature represents the three-dimensional extracellular matrix appropriate for the use as biomimetic scaffolds [3]. In order to achieve a functional tissue construct, the scaffold has to be precisely engineered so as to stimulate specific cells responses [4]. To date many techniques have been established for the fabrication of fibrous scaffolds as ECM alternatives; however electrospinning is considered as one of the successful methods due to its ability to generate fibers of ECM relevance [2, 3].

Most of the conventional electrospinning technique reported for the fabrication of scaffolds lacked of sufficient porosity and spatial interconnectivity. This would inhibit the exchange of nutrients and wastes within the tissue construct [3, 5]. In addition, many tissues including heart, tendon and blood vessel have unique anisotropic structure and architecture that cannot be recapitulated by scaffolds fabricated using the conventional technique [1, 6]. As such, scaffolds fabricated by electrospun fibers with various alignment have shown ability in modeling cell morphology and affecting cell differentiation [4, 5].

In this study an electrospinning auxiliary jig has been designed to facilitate the collection of 3D aligned and random fibrous scaffold as shown in Figure 4.1. The rotating mandrel embedded in the water bath was designed for the collection of 3D aligned scaffold. In order to collect three-dimensional fibrous scaffold, the surface tension of the water bath solution has to be lower than that of the electrospun polymer and electrospinning polymer solution. The Wilhelmy plate method [7] has proven to facilitate the collection of fibrous scaffold where fibres collected from the optimized solution was found to be more uniform without excessive coiling and presented a more porous morphology.

4.2 Design and collection of 3D electrospun scaffold

The electrospinning auxiliary setup designed in this study aims to collect 3D fibrous aligned and random scaffold. The white Teflon bath was used to prevent fibers from attaching to the edges of the bath, while the stainless steel needle-like collector (Fig. 4.1b) was attached to a rotating mechanism for the collection of circumferentially aligned fibres (Fig. 4.1c & d). An advantage of the setup is the flexibility of collecting both random (Fig. 4.1e) and aligned fibres where the circumferentially aligned fibres can be collected on various diameter. As a demonstration, tubular construct with diameter ~ 1 mm and 5mm were made (Fig. 4.1c). The 3D aligned fibers served as a signaling modality for the regulation and maintenance of smooth muscle cells contractile phenotype [8, 9] to be demonstrated in subsequent chapter.

Figure 4.1 Electrospinning auxiliary setup and macroscopic images of scaffold. (a) Front view of Electrospinning auxiliary jig. (b) Top view of Electrospinning auxiliary jig. (c) Macroscopic images of tubular scaffold, showing typical length of scaffold. (d) Cross-sectional view of tubular scaffold, indicating the tunability of scaffold diameter. (e) 3D random scaffold

4.3 Surface tension of bath solution

The bath solution has been optimized using the Wilhelmy plate method [7]. Briefly, in order to form 3D fibrous scaffold structure, fibres have to be deposited in a bath of solution that enhanced deposition and dispersion of fibres rather than a simple deposition on a conventional collector [10]; the conventional collector typically collects fibre of 2D morphology [6, 11]. The bath solution in this context would need to have surface tension value lower than the polymer solution in order to recapitulate 3D scaffold structures [12, 13]. According to equation 1 the surface tension of test liquid can be calculated by assuming $\cos \theta$ as 1 since a platinum plate with high surface energy was used, it is assumed that it could perfectly wet most liquids. Therefore, with the measured wetting force F , the surface tension of test liquid can be calculated.

$$\sigma = \frac{F}{L \cdot \cos \theta} \quad \text{Equation (1)}$$

Surface tension is the key factor for initiating electrospinning process where the electrospinning solution's surface tension has to be overcome by the electric field applied [3]. For a demonstration in this study, the electrospinning solution containing 13 w/v% of PCL in 6.5:3.5 ratio of dichloromethane (DCM) and dimethylformamide (DMF) was found to be 32.44 mN/m. In order to collect 3D fibrous scaffold in the water bath, surface tension of bath solution composition has to be lower than the surface tension of the electrospinning solution [10, 12].

In addition, it has been observed that bath solution has to be at least 6 mN/m lower than the electrospinning solution in order for fibres to be successfully deposited into the bath and get collected on the rotating mandrel. Any bath solution of surface tension greater than 26.44 mN/m would not have fibres entering into the bath. This was clearly demonstrated in our experiment when 50% Ethanol in D.I water, translating to a surface tension of 29.32 mN/m was used, a layer of fibres were found to form on top of the bath solution.

On the other hand, when 70% IPA in D.I water (surface tension of 23.61 mN/m) was used, excessive coiling of fibres has been observed. Similar findings have been reported by Razieh et al. [13] where the increase in surfactant concentration resulted in a decrease in average pore size of scaffold. This could be due to a faster acceleration towards the collector with a reduced surface tension of bath solution [14, 15]. Thus, excessive lowering of surface tension indeed present detrimental effect on fibre morphology.

Henceforth, the bath solution has been optimized to be in the regime of 6 to 9 mN/m lower than the electrospinning solution for the collection of smooth and morphologically presentable scaffold without excessive coiling. For this instance, the bath solution requirement would be 26.44 mN/m where the concentration of 50% IPA in deionized water was found to be suitable for subsequent wet electrospinning process.

Figure 4.2 Surface tension of bath solution mixtures with various content of deionized water (the solvent analyzed include isopropyl alcohol (IPA) and ethanol (ETOH)) Electrospinning solution (PCL) is presented for comparison.

Figure 4.3 Micrographs of 3D random scaffold. (Left) Excessive coiling from incompatible bath solution (70% IPA in D.I water). (Right) Optimized bath solution (50% IPA in D.I water) using Wilhelmy plate method.

Table 4.1 summarized the types of scaffold fabricated using the electrospinning auxiliary setup. In summary, two types of scaffold was fabricated, namely the Polycaprolactone and Poly(lactic-co-glycolic acid) scaffold. Essentially, we are interested to demonstrate the versatility of the newly designed electrospinning setup in addressing the different needs of tissue engineering. The individual scaffold orientation was selected based on the cell culture studies requirement and has been explained in the subsequent chapters.

Table 4.1 Types of scaffold fabricated and tuned according to its cell culture studies requirement

Cell culture studies	Purpose	Scaffold material	Scaffold orientation	Optimized bath solution
Human aorta smooth cells (hSMCs)	Demonstrate phenotypic maintenance due to topographical cues	Polycaprolactone (PCL)	3D Aligned	50:50 isopropyl alcohol (IPA) and deionized water
			3D Random	
Huh7.5 human hepatocarcinoma cell	Micro perfusion studies	Poly(lactic-co-glycolic acid) (PLGA)	Micro 3D Random	70:30 isopropyl alcohol (IPA) and deionized water

4.4 Physical characterization of Polycaprolactone electrospun scaffold

In this chapter, only Polycaprolactone electrospun scaffold has been characterized. This is to demonstrate the effect of fibre alignment on hSMCs function which will be studied in Chapter 6. No physical characterization of Poly(lactic-co-glycolic acid) (PLGA) has been performed since these form of characterization has been established in the group by fellow co-workers [10].

4.4.1 Morphology of Polycaprolactone 3D electrospun scaffold

To assess the morphology of Polycaprolactone (PCL) electrospun fibres, SEM images were captured on PCL 3D aligned and 3D random scaffolds. Figure 4.4 - 4.6 presents the top, cross-sectional view as well as close-up FE-SEM images of the scaffolds respectively. The SEM images revealed that scaffold fabricated on the auxiliary setup demonstrated higher porosity and thicknesses, the 3D aligned scaffold were found to be preferentially aligned along the rotating axis as shown in Figure 4.4a. The fibre thickness and porosity of the aligned scaffold were comparable to the random scaffold, Figure 4.4b.

Figure 4.4 SEM images of Polycaprolactone 3D aligned (a) and random (b) scaffold

Cryo-sectioned images of the scaffold demonstrated that the 3D aligned scaffold, Figure 4.5a, present several layers of preferentially aligned fibres forming a tubular construct. This morphology is similar to the native tunica media layer found in blood vessel. While many other synthetic blood vessels were made from materials like PDMS [16], the absence of

surface topography failed to maintain the phenotypic function of smooth muscle cells which is one of the vital limitation of achieving successful engineered vascular graft [17, 18]. Scaffolds made from the auxiliary setup were found to collect fibrous scaffolds with thickness in the range of 500 – 600 μm (Figure 4.5 a & b) as compared to only 100 – 200 μm when fabricated on conventional setup.

Figure 4.5 Cryo-sectioned SEM images of Polycaprolactone 3D aligned (a) and random (b) scaffold

Close-up images were taken on aligned scaffold (Figure 4.6 a & b) where fibres were found to be smooth with no presence of pits and voids, presenting a consistent substrate for future cell studies.

Figure 4.6 (a) FE-SEM images of Polycaprolactone 3D aligned fibrous scaffold at 10k x and (b) 20k x with smooth and homogenous fibre morphology.

4.4.2 Fibre alignment characterization of Polycaprolactone 3D electrospun scaffold

The alignment of Polycaprolactone fibres were quantified with 2D fast Fourier transform (FFT) by converting the fiber placement from image space to frequency space through an ImageJ® software. This plot consists of grayscale pixels distributed into an outline that can be used to measure the degree of fibre alignment [19, 20]. The frequency pixels were found to be predominately distributed along the vertical axis for 3D aligned scaffold, Fig 4.7a, indicating a preferential alignment. However, the FFT frequency plot for 3D random scaffold, Fig 4.7b, contain frequency pixels that are concentrated in the center with some high frequency pixels placed outside of the origin. An improved contrast of the 2D FFT image is placed in Appendix B for reference. The peripheral frequency pixel correspond to edges, details and noise in the SEM image as a result of the high porosity nature found in 3D scaffolds [19]. The radial intensity summed using an oval-profile plugin was plotted against the angle of acquisition to obtain the FFT alignment plot, Fig 4.7c. The peaks on the FFT alignment plot are narrow and sharply outlined, indicating a good degree of fibre alignment for the 3D aligned scaffold, while the nearly uniform peak height is a hallmark of scaffold consisting of random element as shown in 3D random scaffolds [21]. Such an enhancement in fibre alignment on a 3D format could find potential in the maintenance of smooth muscle cells phenotypic function to be described in chapter 6.

Figure 4.7 2D FFT image and plot of Polycaprolactone 3D aligned & random scaffold. (a) FFT frequency image of PCL 3D aligned scaffold. (b) FFT frequency image of PCL 3D random scaffold. (c) Combined FFT alignment plot.

4.4.3 Scaffold porosity & pore size of Polycaprolactone 3D electrospun scaffold

The porosity and pore size of a scaffold is essential to provide sufficient cellular infiltration and tissue ingrowth [22, 23]. The liquid displacement method has been used to determine the porosity of the scaffold where the porosity of PCL 3D aligned scaffold was found to be $94.09 \pm 0.009\%$ and PCL 3D random scaffold to be $97.75 \pm 0.006\%$, Figure 4.8. Both PCL 3D aligned and random scaffolds were considerably porous, hence permitting cells infiltration and exchange of nutrients.

Figure 4.8 Porosity of Polycaprolactone 3D aligned & random scaffold

The pore size of the two sets of scaffolds were measured on Image J[®] software where the average pore size for PCL 3D aligned scaffold was determined to be approximately $11.63 \pm 3.53 \mu\text{m}$ and PCL 3D random scaffold with $10.623 \pm 3.65 \mu\text{m}$ pore size. Figure 4.9 a & b presents the distribution curve of the measured pore size for the PCL 3D aligned and 3D random scaffold respectively. From these data it can be observed that the electrospinning auxiliary jig enabled the collection of porous 3D aligned and random scaffold which were deemed to be suitable for subsequent cell culture studies.

Figure 4.9 Percentage pore size distribution of PCL 3D aligned & random scaffold.

4.4.4 Contact angle measurement of Polycaprolactone 3D electrospun scaffold

The static water contact angle (WCA) measurement has been performed on PCL 3D aligned and random scaffold to determine the hydrophobicity of the uncoated porous scaffold. Polycaprolactone (PCL) used in this study is inherently hydrophobic where the static WCA of the film was reported to be $137 \pm 2^\circ$ [24]. In this study, the static WCA measured on 3D aligned scaffold was found to be $112.9 \pm 5.3^\circ$ and $107.83 \pm 4.6^\circ$ for 3D random scaffold. These values were significantly lower than the static WCA values reported for PCL film. The effect can be attributed to the excessive pores found in 3D scaffolds where larger pores and porosity formed on the 3D scaffolds promoted the infiltration of water hence reducing the hydrophobic effect of the scaffold [25].

Figure 4.10 Contact angle measurement of PCL 3D aligned and random scaffold

4.4.5 Mechanical properties of Polycaprolactone 3D electrospun scaffold

The average tensile stress of PCL 3D aligned scaffold conducted along the fibre direction was found to be 1183.9 ± 144.48 kPa and 86.08 ± 23.32 kPa for PCL 3D random scaffold. Young's modulus of the aligned 3D scaffold (//) was found to be 16.99 ± 1.17 kPa while that for the 3D random scaffold was found to be 0.2 ± 0.08 kPa. It can be expected that the alignment of fibres in the 3D aligned scaffold contributed significantly to its mechanical properties. In general, the mechanical properties of the material will be significantly enhanced in the direction of alignment. But at the same time it will be much weaker at the transverse direction. This is because more force is required to fail the fibres in the longitudinal direction [26] which then result in significant stiffening in the direction of alignment and increased scaffold anisotropy [27]. Whereas for random scaffold there is no specific directionality of the fibres, rendering the sample isotropic in nature, therefore such material would usually have a lower mechanical properties overall, as compared to fibres tested on the longitudinal direction [28].

In addition, these mechanical values were much lower than many of the reported 2D PCL scaffold data [29, 30]. This is due to the excessive pores formed during wet electrospinning which contributed to a weaker scaffold structure as compared to the reported 2D scaffolds [31]. However, the moduli in this range is relevant for tissue engineering of soft tissues as they are more mechanically compliant [32-34]

Figure 4.11 Tensile stress and Young's modulus plot of PCL 3D aligned (//) and PCL random scaffold. (A) Tensile stress plot. (B) Young's modulus plot.

4.5 Conclusion

In this chapter an Electrospinning auxiliary jig has been designed for the collection of 3D aligned and random scaffold. The fibre alignment was reported to be useful in directing cell migration or the maintenance of cells function, e.g. tendon, heart or blood vessels where the extracellular matrix presents an anisotropic architecture. However, many of the reported electrospinning techniques were fibres on 2D format and they failed to recapitulate the 3D micro-environment of cells. In addition, the three-dimensional architecture was found to better represent the physiological environment of an *in vivo* tissue which will enable the design of an accurate *in vitro* model for drug testing. From the various physical characterization such as pore size, porosity and mechanical properties, it has been shown that 3D aligned scaffold not only had sufficient mechanical integrity, its pore size and porosity would also enable the exchange of nutrients and metabolites. In the following chapter, 3D aligned and random scaffolds made from the established electrospinning technique will be tested on its functionality of cells.

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Chapter 5

Design of a one-step stereolithography (SLA) process for microfluidic perfusion culture

Cells cultured in vitro tend to retain its differentiated phenotype and functions under condition that mimics the natural in vivo environment. Therefore, in this chapter a combinatorial approach has been presented where a PLGA 3D fibrous scaffold was embedded on a microfluidic perfused platform. This is the first instance where a miniaturized 3D fibrous scaffold was embedded on a SLA-printed perfused microfluidic platform. The design of this platform was found to better mimic the in vivo condition for an accurate in vitro model for multiplexing of drug analysis studies.

5.1 Introduction

Microfluidic perfusion culture is highly desirable as it offers a more physiologically relevant environment for cell culturing [1, 2]. The perfusion culture allows controlled delivery and removal of soluble biochemical molecules that mimics many highly perfused organs *in vivo* [3]. Such defined micro-environment are unobtainable in static cultures. The microfluidic culture also enabled precise spatial and temporal control of cellular micro-environment that aids in preserving the phenotypes of cells [4].

While there are many reported articles on microfluidic perfusion culture, most of them were either on 2D monolayer or for 3D cultures that are mainly homogenous hydrogel systems [5]. Since cells cultured on 3D substrate displayed a distinct difference in terms of cell morphology and phenotype [6], in this study we are interested to explore only 3D systems. Additionally, *in vivo* ECMs are usually highly fibrous in nature and therefore here we focus on the incorporation of 3D fibrous scaffold, instead of homogenous hydrogel, on a microfluidic perfusion platform.

The microfluidics platform developed thus far is dominated by poly(dimethylsiloxane) (PDMS) or thermoplastics molding approaches [7-9]. While such devices are biocompatible, transparent and gas-permeable, the fabrication process is considerably labor intensive and the tool making process is expensive with long turn-around times [1]. In addition, the conventional method limits the 3D complexity in the devices produced. Henceforth, 3D printing has attracted much attention for the fabrication of microfluidic devices due to its automation, assembly-free 3D fabrication with high resolution and throughput [10-12].

Among the many 3D printing techniques, Stereolithography (SLA) and Fused Deposition Modeling (FDM) emerged as the most relevant techniques for microfluidics device fabrication [7, 13]. The Stereolithography method is much preferred over FDM as it has higher printing resolution of up to 100 μm channel compared to $\sim 350 \mu\text{m}$ channel for FDM. In addition, SLA has very little topological restriction and FDM can only print circular channel cross-sections [14].

The microchannel fabrication using FDM is considerably challenging since the filament laid down by the extrusion process cannot be joined at the intersections. The structural integrity between the layer is often weak and the size of filaments extruded are larger than the typical channels used in microfluidics [15]. Therefore, in this study Stereolithography is the preferred fabrication process for a perfused microfluidic platform.

In this chapter, we described a one-step Stereolithography process for the fabrication of microfluidic perfusion culture. Briefly, the microfluidic perfusion platform encompasses three distinct layers starting with the first layer on cells visualizing window, layer two encompasses the cell culturing chamber with an embedded 3D fibrous scaffold and the third layer houses the user interface for connecting tubings for media exchange. These three layers were printed on top of each other where after each layer either a PDMS or miniaturized fibrous scaffold would be embedded hence delivering a functional stereolithography printed perfused microfluidic chip.

5.2 Design of 3D microfluidic perfusion culture

The design of 3D perfused microfluidic platform has been described in Figure 5.1. Briefly, Stereolithography method has been adopted to fabricate the 3 layers of perfused microfluidic chip. These 3 distinct layers were directly printed on top of each other and fuses well to form a 3D perfused microfluidic chip. Layer 1 was first printed where the gap was used to house a piece of PDMS upon completion of printing. The purpose of layer 1 was to create a transparent visual window to monitor fluid flow and cellular activity. The second layer was subsequently printed right above layer 1 and upon completion a piece of miniaturized scaffold was placed within the cell culturing chamber and sealed it with another piece of PDMS. The third layer of the perfused microfluidic chip was printed on top of layer 2 where three ferrules were printed for subsequent handling of cell culture media.

Figure 5.1 Design of 3D perfused microfluidic culture

Figure 5.2 Macroscopic image of Stereolithography-printed microfluidic perfused platform

Figure 5.2 presents the macroscopic images of a stereolithography-printed perfused microfluidic chip embedded with a piece of miniaturized PLGA 3D random scaffold. Red dye was added with PBS and perfused to give a clearer image on the channels and cell culturing chamber. Despite many reports on 3D printed microfluidic chip [13-15], this is the first instance where a miniaturized piece of scaffold was embedded. While some reported the use of pillars replicated by replica molding of PDMS on a silicon template, such pillars were used to immobilize cells and create a cell culture compartment [12]. However, among the many reported studies; microfluidic cell culturing platform embedded with a miniaturized ECM mimicking fibrous scaffold is lacking. The presence of a fibrous substrate was adopted to recreate the structural aspects of the native extracellular matrix (ECM). In addition, the 3D fibrous substrate were used to immobilize cells and the porous nature facilitate cell infiltration with the transport of oxygen, nutrients and metabolite waste [6].

5.3 Characterization of 3D perfused microfluidic platform

The physical characterization of stereolithography printed microfluidic chip has been performed to determine the print quality prior to cell culturing studies. The reported manufacturer resolution of the Asiga pico 2 HD in the x - y direction was less than 50 μm and z direction within 1 μm . Generally, the SLA method is capable of printing features with better fidelity which was in concordance to report made by Shallen *et al* [16]. The smallest dimension in the SLA-printed microfluidic platform is 800 μm channel used for delivering perfusion medium to the miniaturized fibrous scaffold as shown in Figure 5.1.

Microscopic images of the PLGA 3D random fibrous scaffold has been taken before and after embedding within the SLA-printed microfluidic platform. The purpose of the microscopic imaging is to ensure that the porous nature of the scaffold remained intact even after perfusion.

5.3.1 Physical characterization of Stereolithography printed microfluidic chip

The dimensional accuracy of the stereolithography printed microfluidic chip were investigated. Figure 5.3 presents the macroscopic images of the individual layer comprising of visualizing window, cell culturing chamber and the printed ferrule. Measurements were taken from ImageJ[®] software, averaged and plotted as shown in Figure 5.4

Figure 5.3 Macroscopic images of the individual SLA printed layers. Scale bar = 0.5 cm.

Figure 5.4 Summary of measurement taken on 3 different SLA-printed layers

From Figure 5.4 it can be observed that the Stereolithography method is capable of replicating the microfluidic platform accurately with the optimized process parameter. In general, all dimensions were replicated within 5% accuracy indicating that the SLA method is a good choice for fabricating such perfusion platform. However, the surface finishing of the printed prototype is not as ideal as those of casting [3, 12] or injection molded parts [17]. Nevertheless, SLA offers a quick solution to determine the functionality of the perfusion platform obviating the need for excessive secondary processes such as bonding of top and bottom layers, punching of inlet and outlet for medium perfusion [18].

5.3.2 Effect of perfusion on embedded scaffold

The effect of perfusion on PLGA 3D random miniaturized scaffold embedded on a SLA-printed perfused microfluidic chip has been investigated. Figure 5.5a presents the SEM image of a miniaturized PLGA 3D random scaffold prior to embedding on the SLA-printed perfused microfluidic chip. This microscopic image served as a control to the effect of perfusion. It can be observed from the microscopic image that scaffold fabricated by the optimized wet electrospinning method (described in Chapter 3) presents a generally porous structure where the average pore size measured by the ImageJ software is approximately $15.12 \pm 3.91 \mu\text{m}$. More than 50% of the pore falls within the range of 12.5 to 15 μm indicating a sufficiently large distribution of pores which could facilitate cells infiltration.

Figure 5.5 Effect of perfusion on PLGA 3D random miniaturized scaffold. (a) Fibrous scaffold prior to embedding. (b) Percentage pore size distribution of scaffold prior to embedding. (c) Fibrous scaffold after embedding on SLA-printed perfused platform. (d) Percentage pore size distribution of scaffold after embedding on SLA-printed perfused platform.

Figure 5.5c presents the microscopic image of a miniaturized fibrous scaffold after embedding on the SLA-printed perfused platform. The printed device was immersed in 70% ethanol for 30 mins prior to two rounds of 10ml of 70% ethanol flushing to remove any residual resin. The device is subsequently freeze-dried overnight to remove residual solvent. From the SEM image, scaffold after multiple cleaning presents a flatter structure as compared to the scaffold prior to embedding. However, the average pore size of the embedded scaffold is approximately $15.87 \pm 3.59 \mu\text{m}$, similar to the scaffold prior to embedding. The pore size does not seem to be affected by the multiple ethanol rinsing, although more than 40% of pore size falls within 15 to $18 \mu\text{m}$. There is no statistical difference between this two groups of scaffold.

The effect of media perfusion has been investigated where Figure 5.6a presents the microscopic image of PLGA 3D random miniaturized scaffold after 4 days of media perfusion. The average pore size of the perfused scaffold is approximately $13.34 \pm 2.60 \mu\text{m}$, presenting approximately $2 \mu\text{m}$ decreased in average pore size as compared to scaffold prior to embedding ($15.87 \pm 3.59 \mu\text{m}$). However, such a difference did not affect the subsequent Huh7.5 cells viability studies on a perfusion platform. A detailed z-stack and cross-sectioning will be performed to determine the cellular infiltration of the scaffold.

Figure 5.6 Effect of perfusion on PLGA 3D random miniaturized scaffold. (a) Fibrous scaffold after 4 days of media perfusion. (b) Percentage pore size distribution of scaffold after 4 days of media perfusion.

5.4 Optimization of perfusion setup

Shear stress is known to have detrimental effect on the cellular phenotype and functions [4, 14]. However, it is also an inherent effect of a perfusion culture system, therefore it is imperative to design and operate the perfusion culture where the applied shear stress is several magnitude below the adverse value. The objective of this study is to develop a miniaturized liver perfusion platform for the multiplexing of drug analysis, hence hepatocyte derived cellular carcinoma cell (Huh 7.5) were used as model cells. The suitable range of shear stress that does not cause any detrimental effect on the primary liver hepatocytes were reported to be less than 0.2 Pa [4, 19].

The computational fluid dynamic (CFD) simulation, Flow 3D, was used to estimate the corresponding shear stress at the cell culturing chamber and along the micro channel. Figure 5.7 presents the CFD simulation result of shear stress with varying channel dimension. The volumetric flowrate used in the simulation is 18 ml/h with chamber porosity of 85%. The detailed simulation parameters have been reported in Chapter 3.

Figure 5.7 Effect of channel dimension on fluid shear stress. (a) Channel width 300 μm . (b) Channel width 600 μm . (c) Channel width 800 μm .

The shear stress experienced by the wall is governed by a simple 2D Poiseuille flow system described in equation 2:

$$\tau = \frac{6\mu Q}{h^2 w} \quad (2)$$

where, μ defines viscosity of fluid, Q as flowrate, h as channel height and w as channel width.

From the CFD simulation results shown in Figure 5.7, as the channel width decreases the shear stress increases significantly, this is in concordance to the equation described above. While the volumetric flowrate used in this study was fixed at 18 ml/h, the actual flowrate used is much lower. However, the simulation served as a guideline for the perfusion culture development and in this case channel width of 800 μm exhibited acceptable shear stress

along the channel width and within the culturing chamber. Therefore 800 μm channel width dimension will be used for subsequent cell perfusion studies. The flow profile of various flowrate has been attached in Appendix C for reference and the optimized perfused parameters exhibited laminar flow within the micro-perfused platform.

It is also worth noting that the cell culturing chamber did not present excessive shear stress despite having a high perfusion flow rate. This could be attributed to the comparatively large cell culturing volume which in turn reduces the shear stress.

The Forchheimer saturated drag model has been used to relate the pressure loss in porous medium where the PLGA 3D fibrous scaffold was placed. In Forchheimer's equation, pressure drop is define as [20, 21]:

$$-\nabla p = (A + B|\mu_{bulk}|)\mu_{bulk} \quad (3)$$

where A and B are coefficients defined by experimental data, μ as the apparent velocity and ∇p is the pressure gradient in real space within the porous medium.

$$A = 180 / D_{fiber}^2 \quad B = 2.0 / D_{fiber}$$

The average fiber diameter of the scaffold used in the Flow3D simulation was measured on ImageJ and found to be $0.95 \pm 0.038 \mu\text{m}$. Appendix C presents the effect of porosity on shear stress in the cell culturing chamber.

A summary of the simulation data is presented in Figure 5.8 where the effect of channel width and flowrate has been summarized. As expected, as channel width decreases, shear stress increases beyond the safe operating limits of 0.1 Pa. Similarly, as flow rate increases shear stress increases. Hence for all future perfused microfluidic platform, channel width of 800 μm along with perfusion flow rate of 0.12 ml/h will be used. This is to ensure that the device is operating within the allowable shear stress for the culturing of hepatocytes reported to be in the range of 0.1 Pa.

Figure 5.8 Effect of (a) channel width simulated at 18 ml/h and 85 % porosity and (b) flowrate simulated at 800 μm and 85 % porosity on shear stress

5.5 Illustration of perfusion setup

The microfluidic perfusion culture has been setup as shown in Figure 5.9. A six well plate has been used to hold the SLA-printed perfused microfluidic platform where a syringe pump was used to infuse culture medium at a controlled rate. The perfusion culture was placed in an incubator where cells are cultured at 37°C and 5% CO_2 .

The medium outlet which is opposite to the perfusion inlet will be collected for subsequent cell culture analysis, such as Albumin ELISA. The third inlet port was sealed by a paper clip after the infusion of cells. The current setup has proven to be sterile and is a viable method for the culturing of Huh 7.5 liver hepatocytes cells on a miniaturized perfused platform.

Figure 5.9 A schematic illustration of the miniaturized microfluidic perfusion setup

5.6 Conclusion

A one-step stereolithography (SLA) process for the fabrication of miniaturized microfluidic perfusion platform has been developed in this study. A miniaturized PLGA 3D random fibrous scaffold was embedded in the perfused culture to recreate the structural aspect of the native extracellular matrix (ECM) and to immobilize Huh7.5 liver hepatocytes cells. The physical characteristic of the perfused platform has been quantified where dimensions were found to be within $\pm 5\%$ error. An actual Z-stack confocal microscopy and cross-sectioning of the scaffold has been conducted and will be reported in the subsequent chapter to analyze cells infiltration into the scaffold. Computational Fluid Dynamic (CFD) simulation, Flow3D, has been used to optimize the perfusion culture setup. Since the microfluidic perfusion device has to be operated within 0.1 Pa to avoid any detrimental effect on cells, a channel width of 800 μm and perfusion flow rate of 0.12 ml/h were determined and will be used for subsequent cell culture work. From the initial microfluidic perfusion setup, no contamination has been observed and the device proved to be suitable for subsequent cell viability studies.

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Chapter 6

A demonstration of cell viability and function maintenance on miniaturized 3D perfused microfluidic platform and 3D aligned fibrous scaffold

The cell viability of the newly designed Stereolithography-printed perfused micro-platform was evaluated. Huh7.5 hepatocellular carcinoma cells were used as model cells. A series of cell culture studies were performed on the micro-perfused platform where enhanced cell infiltration and upregulation of Albumin and CYP3A cytochrome has been observed. In addition, 3D aligned scaffold collected on the auxiliary Electrospinning jig were used as topographical cues for human aorta smooth muscle cells (hSMCs). SMCs cultured on 3D aligned scaffold exhibited definitive α -SMA and MHC markers which was not observed in 3D random counterpart. Taken together, the newly developed technologies have demonstrated sufficient cell viability and function maintenance. A combinatorial approach can be put together for future application studies.

6.1 Introduction

Cells are highly sensitive to its surrounding and the eventual cell fate is determined by its interaction with neighboring cells or surrounding environment and substrates [1]. Therefore, with this consideration, a 3D culture model may better represent the physiological environment of cells and hence facilitate the preservation of a functional tissue. Such a biologically-relevant *in vitro* model is highly desirable since the *in vivo*-like phenotypes of cells allows the extraction of meaningful cell-based assays for drug testing applications [2].

In this study, a miniaturized 3D perfused microfluidic platform has been developed to study the effect of perfusion on 3D fibrous scaffold. The 3D fibrous scaffold fabricated by Electrospinning is highly porous and was embedded in the micro-perfused platform. This is the first instance where a miniaturized PLGA 3D random fibrous scaffold was embedded on a Stereolithography-printed perfused microfluidic platform. The primary goal of this work is to establish the feasibility of miniaturized 3D bio-mimicking fibrous scaffold by demonstrating the viability and ability to retain functional properties of Huh7.5 liver hepatocellular carcinoma cells on the perfused system. The conventional hepatocytes cultures were mainly static of 2D format [3] or 3D cells agglomerates [4, 5] which failed to recapitulate the fibrous ECM and highly perfused nature of a liver organ. Therefore, the development of a miniaturized 3D perfused microfluidic platform would better mimic the *in vivo* environment thus presenting a more predictable drug screening platform.

A part of the work lies in the development of an Electrospinning auxiliary jig for the collection of 3D aligned and random scaffold. The purpose of the jig is to collect both highly porous aligned and random scaffold to mimic the different tissues' extracellular matrix (ECM) [6, 7]. For example, human aorta smooth muscle cells were supported on circumferentially aligned ECM *in vivo* [8, 9]. Conventional aligned fibrous scaffold were mainly of 2D nature however the tunica media layer of blood vessel cells required several layers of circumferentially aligned smooth muscle cells to provide structural integrity [10, 11]. Henceforth, in this study 3D aligned fibrous scaffold were used as topographical cues

to align and maintain the phenotype of vascular smooth muscle cells (SMCs) which were reported as one of the vital limits for successful graft replacement [12, 13].

6.2 Cell viability studies on miniaturized 3D perfused microfluidic platform

6.2.1 Cell seeding efficiency & cell proliferation

Cell seeding efficiency was performed on 3D-printed perfused microfluidic platform to determine the efficient number of cell density to be seeded. Three different cell density of Huh 7.5 human hepatocarcinoma cell line etc., 50k, 800k and 1.5mil were seeded on the platform and allowed to be incubated for 16 hours. Figure 6.1 presents the seeding efficiency for 3D miniaturized scaffold on micro-perfused platform (3DR/Perfused).

Figure 6.1 (A) Cell seeding efficiency plot for PLGA 3D random scaffold embedded on micro-perfused platform (3DR/Perfused). (B) Proliferation of Huh7.5 cells on TCPS, 3DR/Static and 3DR/Perfused culture at Day 1 and Day 4 respectively.

It has been observed from Figure 6.1a that the seeding efficiency at 50k is at its optimal for the micro-perfused platform. As the number of cells seeded increase further, the scaffold was unable to provide sufficient space for the cells to adhere, therefore the cell seeding efficiency declined significantly. Hence, for future cell studies, the seeding density was kept at 50k per well or device.

The proliferation assay CCK8 was performed on various substrates to evaluate the effect of micro-perfusion. The results showed that cell number increased significantly after 4 days of culture. The cell number between 3DR static and 3DR perfused did not exhibit any significant difference indicating the 3D scaffold system supported the proliferation of Huh7.5 cells with or without perfusion.

6.2.2 *Cell viability on miniaturized 3D perfused microfluidic platform*

To investigate the cell viability of hepatocytes seeded on the SLA-printed perfused microfluidic platform, a LIVE/DEAD assay has been performed after four days of culture. Figure 6.2 presents the confocal Z-stack images of the various substrates used for culture. From the images, Huh7.5 cells cultured on the TCPS plate presents the least amount of dead cells compared to 3DR/Static and 3DR/Perfused culture. This is much expected since there is direct medium exchange on the surface of the 2D culture. On the other hand, 3DR/Static culture presents the most amount of dead cells since on a static culture there bound to be some regions with limited exchange of nutrients. The condition was much improved when perfusion was introduced to the miniaturized 3D random (3DR) scaffold. Although there were still dead cells, they were of lesser quantity and more distributed compared to the static culture.

The merged Z-stack confocal images presented the three-dimensional scan of Huh7.5 cells cultured on the respective substrate. The TCPS presents a relatively thin monolayer of cells where the thickness found on the confocal Z-stack images was approximately 60 μm . The 3DR/Static culture which comprises of a miniaturized piece of porous random substrate saw a relatively good cells infiltration after 4 days of culture. Cells were found to lie within the scaffold of approximately 200 μm thickness from its surface.

The perfusion culture presents a thicker cell construct with more than 400 μm of cell depth observed. The cells were found viable at such a thickness due to the perfusion of medium, which delivers the necessary nutrients to the core of the tissue construct. A cryo-sectioning of the scaffold 3DR/Static and 3DR/Perfused will be presented in the following section to have a better presentation of the amount and depth of cells infiltrated.

Figure 6.2 Cell viability test of Huh7.5 hepatocellular carcinoma cells on TCPS (LEFT), 3DR/Static culture (CENTER) and 3DR/Perfused culture (RIGHT). Confocal Z-stack images after 4 days of culture. The live cells were stained with Calcein Am (green) and dead cells were stained with Ethidium homodimer-1 (red) respectively.

6.2.3 *Cell infiltration studies*

Cryo-sectioning was performed on 3DR/Static and 3DR/Perfused scaffold to investigate the amount and depth of cells infiltrated. Figure 6.3 presents the fluorescence microscopy images of the cryo-sectioned samples. From the images, 3DR/Perfused scaffold presented higher cell infiltration with up to a depth of 400 to 500 μ m from scaffold surface. On the other hand, cells infiltration for 3DR/Static was found to be approximately 200 μ m from scaffold surface.

A higher cell infiltration on a perfusion culture suggests that cells were better supported on the perfused platform. The perfusion allows the exchange of nutrients and metabolites which is vital for healthy cells growth [2]. The 3D porous scaffold embedded on the SLA-printed perfused microfluidic platform have also proved to be capable of supporting the adhesion of hepatocytes and facilitate the exchange of medium due to its porous nature. While there are also similar reports indicating sufficient cells infiltration on 3D porous scaffold [14, 15], the perfusion platform developed in this study provides the recapitulation of the physiological micro-environment and controlled the distribution of biochemical molecules and shear stresses at cellular level [2].

The depth of cells infiltration on 3DR/Static scaffold were found to be lower compared to the perfused platform. This is much expected since most of the refreshed nutrients reside on the scaffold surface as compared to the perfusion culture. The cells on the static culture will gradually infiltrate into the scaffold only when the scaffold surface has been taken up [16]. However, for perfused culture, medium were constantly being refreshed throughout the culture platform. Such micro-perfusion allows the exchange of medium and metabolites even at the center of a thick tissue construct, thereby promoting better cell infiltration and function when compared to a static platform [5, 17].

Figure 6.3 Cryo-sectioned images of miniaturized PLGA 3D random scaffold cultured on Static (3DR/Static) and SLA-printed perfused (3DR/Perfused) culture at Day 4. The nuclei of Huh7.5 cells were stained with DAPI (blue). The red outline indicates the surface of the scaffold. (Scale bar = 100 μ m).

6.2.4 Gene expression & Albumin secretion test

The gene expression of Albumin and CYP3A cytochrome was performed to examine the function of Huh7.5 cells cultured on the various substrate. Albumin is the most abundant protein produced in the liver and it is a widely accepted marker of hepatocyte synthetic function [18]. Albumin synthesis occurs exclusively in the liver and it participates in the intermediary drug metabolism. Cytochrome P450 enzymes are essential for the metabolism of drugs and endogenous materials [19]. The CYP3A subfamily has been selected in this study since it contributes to the biotransformation of more than 50% of oxidatively metabolized drugs [20].

The gene expression presented in Figure 6.4 were normalized to TCPS culture. At day 4, the expression of Albumin was approximately 3 times for 3DR/Perfused culture and relatively marginal expression for 3DR/Static culture. The Albumin gene expression were found to be significantly higher as compared to the other two substrate indicating the perfused culture is favorable for the culturing of hepatocytes since the liver is a highly perfused environment [5].

Figure 6.4 Relative gene expression analysis of Huh7.5 hepatocellular carcinoma cells at Day 4. Relative gene expression levels (e.g., $\Delta\Delta C_T$ were normalized to TCPS samples). The asterisks indicate statistical significance where * indicate $p < 0.05$ and ** indicate $p < 0.01$ respectively.

Among the various isoforms for Cytochrome P450, CYP3A4 is the most abundant and widely studied enzyme because it metabolizes more than half of the administered drugs while the CYP3A7 is often considered a fetus-specified enzyme [19]. In this study significant upregulation of CYP3A7 has been observed for 3DR/Perfused culture (~3.4 fold), while the 3DR/Static culture did not exhibit any enhancement. The expression of CYP3A7 was significantly lower for the 3DR/static culture compared to the other substrates. Although CYP3A7 is often considered a fetus-specified enzyme, there were studies reported on a subset of adult human livers expressing significant levels of CYP3A7 and some of which carried the CYP3A7*1C allele containing element of CYP3A4 [21].

The CYP3A4 function was examined on the three substrates where 3DR/Static culture did not express any function for this enzymes. The lack of CYP3A4 function could be an indication of poor liver function on a static platform [19, 20]. There is no statistical difference between TCPS and 3DR/perfused culture for CYP3A4 enzyme. Taken together, expression of Albumin and CYP3A enzymes were found to be upregulated or maintained near nominal for the perfusion culture. Although in this case the CYP3A7, often related to

a fetus-specific function [19], were found to be more upregulated than the CYP3A4 enzymes; these phenomena have already been reported in a subset of adult livers, indicating such possibility where these livers expressed significant levels of CYP3A7 and carried the CYP3A7*1C allele of CYP3A4 element [21]. A further quantification of Albumin secreted on these substrates will be studied to determine the effectiveness of the perfusion culture.

The quantification of Albumin synthesis was performed on 3DR/Static and 3DR/Perfused culture. Medium from the static culture and perfusate from the perfused culture was collected at day 4 where the amount of Albumin was quantified on an ELISA kit [22]. Figure 6.5 presents the Albumin quantity at day 4 where the perfused culture had significantly higher amount of Albumin produced per million cells. This indicates a more functional hepatocyte culture on a perfusion platform.

Figure 6.5 Quantification of Albumin synthesis for 3DR/Static and 3DR/Perfused culture at Day 4, normalized to cell number quantified by cck8 assay. The asterisks indicate statistical significance where * indicate $p < 0.05$.

6.3 Cell morphology and phenotypic maintenance of human aorta smooth muscle cells (hSMCs) on 3D aligned scaffold

Apart from the perfused system, we have also developed a 3D aligned fibrous scaffold as another proof of concept of the importance of 3D architecture in inducing and maintaining cellular phenotypes. It has been widely reported that topographical cues e.g., alignment of fibers can direct cell orientation, proliferation and differentiation [15, 23]. In this study, the use of surface topographical cues to align and maintain the phenotype of human smooth muscle cells (hSMCs) has been proposed since it is reported as one of the vital limitation for successful graft replacement [7, 24]. The auxiliary electrospinning setup developed in this study is capable of collecting circumferentially aligned fibres on a 3D tubular format, this micro-architecture was found to be similar to the tunica media layer of blood vessel [12, 25]. The presence of aligned fibres served as a signaling modality to induce cell alignment and the maintenance of contractile phenotype [25, 26]. Hence, human aorta smooth muscle cells (hSMCs) seeded on the fabricated 3D aligned fibres were analyzed for the cell proliferation, morphology and phenotypic maintenance through immunofluorescence staining studies.

6.3.1 Cell proliferation and F-actin staining for cytoskeleton structure

Cell proliferation and F-actin staining for cytoskeleton studies were assayed on the respective substrate to evaluate the effect of hSMCs on different substrate topography. The results showed that cell numbers increased significantly for 3D aligned scaffold after 7 days of culture ($p < 0.01$) indicating a higher proliferation ability, Figure 6.6a. The cell number however remained relatively constant after 7 days of culture for both 3D aligned and 3D random scaffold. A further observation on the F-actin staining shown in Figure 6.6b and 6.6c indicates that 3D aligned scaffold supported the growth of an elongated shape (indicated by long, one-directional F-actin) cytoskeleton with preferential cell alignment along fibre axis. Such a cell-shape directionality is similar to the native SMCs found in vascular media layer [12, 25] it also indicates the contact guidance effect of the underlying substrate supporting cells alignment along fibre axis [27]. hSMCs grown on 3D random

scaffold however did not show aligned and long actin filament indicating round cell morphology after 21 days of culture, signifying a lack in the ability of promoting a contractile phenotype [8, 27, 28]. Since promoting of well-differentiated contractile SMCs phenotype is one of the approaches in minimizing the development of vascular pathologies [25, 29, 30], cells grown on 3D aligned scaffold presents an alternative to maintain the contractile phenotype of hSMCs.

Figure 6.6 Human SMCs proliferation and morphology studies on 3D aligned and 3D random scaffold (as denoted by 3DA and 3DR respectively). (a) Proliferation of hSMCs on 3D aligned and 3D random scaffold at 1, 4, 7 and 14 days of culture. * $p < 0.01$. (b) Fluorescence images of hSMCs

on 3D aligned and (c) 3D random scaffold after 1, 7 and 21 days of culture. The actin filaments and nuclei of hSMCs were stained with Rhodamine Phalloidin (red) and DAPI (blue) respectively. (Scale bar = 50 μm). White lines at top left hand of 3D aligned images denote fibre alignment.

6.3.2 Phenotypic maintenance of hSMCs on 3D aligned scaffold

Further cell studies were conducted to determine the phenotype of hSMCs in *in-vitro* culture. The characteristic of contractile protein expression in hSMCs is the presence of alpha smooth muscle actin (α -SMA) and smooth muscle myosin heavy chain (MHC) [30-32]. While α -SMA is the first known protein expressed during the early stages of SMCs differentiation, it is less definitive [24]. MHC on the other hand is a late protein marker during hSMC development and it serves as an exclusive marker to identify SMC specificity [25, 30]. Figure 6.7 presents the immunofluorescent staining images of scaffold seeded with hSMCs after 21 days of culture. hSMCs cultured on 3D aligned fibrous substrate (top row) were found to exhibit definitive α -SMA and MHC markers that are orderly organized as microfilament bundles. This result suggest that 3D aligned fibrous scaffold with the expression of late MHC markers promoted the maintenance of hSMCs contractile phenotype *in vitro* [12, 33]. On the other hand, a relatively low degree of protein expression and irregularity has been observed on 3D random scaffold (bottom row). From the immunostaining result, hSMCs appear to be in their synthetic phenotype since there were limited expression of MHC late marker. This observation is consistent with the F-actin cytoskeleton staining results where hSMCs cultured on 3D random scaffold failed to exhibit the typical spindle elongated contractile filament bundle. Therefore, the positive expression of α -SMA and MHC on 3D aligned fibrous scaffold suggest that hSMCs were able to retain its functionality on such *in vitro* culture.

Figure 6.7 Immunofluorescent staining images of α -SMA and MHC expressions of human SMCs after 21 days of culture. Cells were stained using mouse monoclonal anti alpha smooth muscle actin (α -SMA) (green), rabbit polyclonal anti smooth muscle myosin heavy chain (MHC) 11 (red), and DAPI (blue). (Scale bar = 100 μ m).

6.4 Conclusion

Two sets of cell culture studies were performed and presented in this chapter. For the first set, Huh7.5 hepatocellular carcinoma cells were used as model cells. The cell viability and function maintenance has been demonstrated on the newly designed Stereolithography-printed perfused microfluidic chip. A miniaturized piece of PLGA 3D fibrous random scaffold was embedded in the SLA-printed micro-perfused platform where cells were embedded, maintained and perfused. From the cell culture studies, 3DR/Perfused platform demonstrated sufficient cell viability. The cells were found to infiltrate more than 400 μ m into the scaffold at day 4 for perfused culture but less than 200 μ m on a static culture. The gene expression of Albumin and CYP3A cytochrome was found to be much upregulated compared to its static counterpart. Taken together, the perfusion culture has proven to be a physiologically-relevant platform for the maintenance of hepatocytes that could be fine-tuned further for future drug screening studies.

In the second study, 3D aligned scaffold fabricated by the newly designed Electrospinning auxiliary jig was used to demonstrate the phenotypic maintenance through topographical cues. In this study, human aorta smooth muscle cells (hSMCs) were used as model cells. The cytoskeleton of SMCs seeded on such substrate exhibited preferential alignment along fibre axis. Such a cell-shape directionality is similar to the native SMCs found in vascular media layer. Immunofluorescence staining suggest that SMCs cultured on 3D aligned scaffold exhibited definitive α -SMA and MHC markers which served as an exclusive marker for SMC specificity. Hence, the collective results suggest that hSMCs were able to retain its functionality on 3D aligned scaffold which was not observed for 3D random counterpart.

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Chapter 7

Conclusion & Future Work

This chapter summarizes the development and results obtained in order to achieve the objectives of the dissertation. In general, cell viability, proliferation and gene expression for Huh7.5 cells have been studied on the miniaturized micro-perfused platform. The 3D aligned electrospun scaffold were used as surface topographical cues to demonstrate the phenotypic maintenance of human aorta smooth muscle cells. While each of the cell culturing activities demonstrated the purpose of the developed platform; a combinatorial approach can be put together for future applications (e.g. 3D aligned scaffold on perfusion culture).

7.1 Conclusion

The objective of this dissertation was to develop a miniaturized micro-perfused platform where 3D fibrous scaffold could be embedded to provide a more physiologically-relevant micro-environment for cell culture studies. The fibrillary and porous nature of the embedded scaffold were thought to recapitulate the structure aspect of the extracellular matrix (ECM) of a native tissue. Cells cultured on a perfused platform is highly desirable since it allows the controlled delivery and removal of soluble biochemical molecules that mimics highly perfused organs *in vivo* such as liver. The resultant platform will therefore present a physiologically relevant micro-environment suitable for the multiplexing of drug analysis.

In order to realize the idea and design of the platform, an Electrospinning auxiliary jig has been designed. The newly designed Electrospinning jig is capable of collecting 3D aligned and random scaffold through the optimization of bath solution using the Wilhelmy plate method. To collect three-dimensional porous scaffold, the surface tension of bath solution has to be 6 to 9 mN/m lower than the electrospinning solution. This will ensure the collection of smooth and morphologically presentable scaffold. The collected scaffold would then be embedded on a micro-perfused platform for further cell culture studies.

The Stereolithography process was adopted for the fabrication of micro-perfused platform for its ease and quick turn-around time on various design iterations. A one-step Stereolithography process has been developed where the 3D fibrous scaffold was embedded without secondary bonding. Conventional assembly of the microfluidic platform is considerably tedious and inefficient since it requires pre-treatment of the microfluidic chip followed by the bonding process. Therefore, the design of such process is efficient since the resultant platform could be used immediately after sterilization obviating any tedious secondary processes.

A series of physical characterization has been performed on the electrospun scaffold and micro-perfused platform. Briefly, both PCL 3D aligned and random scaffold exhibited more than 90% porosity where the average pore size was found to be $11.63 \pm 3.53 \mu\text{m}$ on 3D aligned scaffold and $10.623 \pm 3.65 \mu\text{m}$ on 3D random scaffold. Both scaffold demonstrated sufficient thicknesses within the range of 500 – 600 μm . From these physical data, the electrospun scaffold presents suitable pore size and porosity for culturing of cells.

The dimensional accuracy of the Stereolithography-printed perfused microfluidic platform was investigated. In general, all dimensions were replicated within 5% accuracy indicating that the SLA method is a good choice for fabricating such perfusion platform. The effect of perfusion on 3D miniaturized scaffold embedded on a SLA-printed perfused microfluidic chip has been investigated. Scaffold after multiple cleaning using 70% ethanol presents a flatter structure as compared to the scaffold prior to embedding. However, the average pore size of the embedded scaffold is approximately $15.87 \pm 3.59 \mu\text{m}$, similar to the scaffold prior to embedding. The pore size does not seem to be affected by the multiple ethanol rinsing, although more than 40% of pore size falls within 15 to 18 μm .

The effect of media perfusion has been investigated on the miniaturized scaffold after 4 days of continuous media perfusion. The average pore size of the perfused scaffold is approximately $13.34 \pm 2.60 \mu\text{m}$, presenting approximately 2 μm decreased in average pore size as compared to scaffold prior to embedding ($15.87 \pm 3.59 \mu\text{m}$). Such a phenomenon is expected since the scaffold has experienced multiple washes and may have slightly collapsed. In addition, such a difference did not affect the subsequent Huh7.5 cells viability studies on a perfusion platform.

The computational fluid dynamic (CFD) simulation, Flow 3D, was used to estimate the corresponding shear stress at the cell culturing chamber and along the micro channel. Since hepatocytes are highly shear-sensitive, the suitable range of shear stress that does not cause any detrimental effect on liver hepatocytes were reported to be less than 0.2 Pa. From the Flow3D simulation studies, channel width of 800 μm and perfusion flowrate of 0.12ml/h will be used since it operates within the allowable shear stress.

Huh7.5 hepatocellular carcinoma cells were used as model cells to examine the cell viability and function maintenance of the newly fabricated 3D perfused platform. From the cell proliferation and LIVE/DEAD assay, 3D perfused culture presents sufficient cell viability as compared to TCPS and 3D static culture. On the perfused culture, cells were found to infiltrate more than 400 μ m into the scaffold at day 4, but less than 200 μ m on a static culture. The gene expression of Albumin and CYP3A cytochrome was found to be much upregulated, ~3 times and 3.4 times respectively as compared to its static counterpart. The Albumin synthesis of the perfused culture was found to be 3 times more than the static culture. Therefore, the perfusion culture has proven to be a physiologically-relevant platform for the maintenance of hepatocytes for future drug application studies.

The following cell culture studies were performed to demonstrate the effect of surface topographical cues on the maintenance of cells phenotype. Human aorta smooth muscle cells (hSMCs) were used as model cells in this study. The actin filament of SMCs seeded on such substrate exhibited preferential alignment along fibre axis. Such directionality is similar to the native SMCs found in vascular media layer. Immunofluorescence staining suggest that SMCs cultured on 3D aligned scaffold exhibited definitive α -SMA and MHC markers which served as an exclusive marker for SMC specificity. Hence, the collective results suggest that hSMCs were able to retain its functionality on 3D aligned scaffold which has not been observed for 3D random counterpart.

Taken together, the newly developed technologies have demonstrated good support for cell viability and function maintenance where a combinatorial approach can be put together for future application studies.

7.2 Future Work

7.2.1 *Extension of Huh7.5 cell culture period*

The current micro-perfused platform cultured with Huh7.5 cells were maintained for 4 days prior to a series of cell studies. The purpose of this short span of cell culturing time was [1] to examine the cell viability on such perfused microfluidic platform. It is the first instance where a miniaturized piece of 3D fibrous scaffold was embedded on a Stereolithography-printed perfused microfluidic platform.

Although results on cell viability, proliferation and various CYP3A function proved to be positive, the short span of cell culturing time is not representative of an accurate *in vitro* tissue model for drug testing application [1]. Henceforth, it is of great interest to extend the culture for at least 14 days to have a better representation of a liver tissue model where meaningful biology data can be extracted for drug testing applications [2].

7.2.2 *Drug testing on micro-perfused platform*

The primary goal of this study was to develop a physiologically relevant *in vitro* liver tissue model for drug testing application. With the extension of cell culturing period, meaningful data can be extracted from the analysis of anti-cancer drugs or drugs toxicity studies. Although various cell culturing platform has been developed for drug analysis [3-5], the use of ECM-mimicking substrate on a micro-perfused platform has not been reported.

Therefore, with an extended perfused micro-liver tissue platform, the functional micro-liver tissue could be exposed to various drugs. To start off, hepatotoxicants such as Acetaminophen, Diclofenac or Rifampin could be infused to determine the hepatotoxicity profile on the micro-liver tissue platform.

7.2.3 *Redesign of micro-perfusion culture*

The current design of micro-perfused platform allows the culturing of hepatocytes on a single chamber. Such a design is useful for a quick proof-of-concept; however, it defeats the advantages of utilizing microfluidic platform where cell culturing activities can be easily multiplexed with various integrated systems. The micro-perfused platform could be designed to house a few cell culturing chamber and setup to introduce various drugs at multiple timepoint. The material of current platform could also be changed to a more thermally stable yet biocompatible such as silicon, Poly(methyl methacrylate) or Polycarbonate for prolonged cell culture studies.

7.2.4 *Determine the effect of perfusion on hSMCs cultured on 3D aligned scaffold*

The current cell culture studies on human aorta smooth muscle cells (SMCs) was performed on 3D aligned fibrous scaffold where definitive bundles of α -SMA and MHC markers have been observed. However, the current studies was conducted on a static platform which is not a physiological-mimicking environment for blood vessels. Therefore, a perfusion culture which incorporate 3D aligned fibrous scaffold would be interesting to explore. The additional perfusion on SMCs should bring about greater physiologically-mimicking environment of blood vessel.

Figure 7.1 presents a schematic of how vascularization could be incorporated on organ-on-a-chip platform to provide a more physiologically relevant *in vitro* model for multiplexing analysis [6].

Figure 7.1 Schematic of conceivable vascularized platform for 3D micro-physiological system [6].

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Appendix A

Detailed dimensioning of micro-perfused platform.

Appendix B

An improved contrast of the 2D FFT plot is placed in underneath the original image for comparison.

Figure B1 2D FFT image of Polycaprolactone 3D aligned & random scaffold. (TOP) original 2D FFT image. (BOTTOM) improved contrast of 2D FFT image.

Representative stress-strain curve of the PCL 3D aligned and random scaffold has been included for reference.

Figure B2 Stress vs strain curve of (LEFT) PCL 3D aligned scaffold and (RIGHT) PCL 3D random scaffold

Appendix C

CFD simulation on the effect of shear stress from various parameters.

Effect of flowrate on shear stress:

- Channel width: 800 μm
- Chamber porosity: 85%; Drag Coefficient A – $1.8\text{e}+11 \text{ 1/m}^2$; Drag Coefficient B – $1.8\text{e}+05 \text{ 1/m}$

P.S: Any volumetric flow rate lower than 18 ml/hr would require long simulation hours.

Flow rate (ml/h)	50 % filled	100 % filled
360 ml/h
180 ml/h

From the above simulation it can be seen that as flowrate increases the shear stress experienced at channel wall increased significantly. While flowrate of 18 ml/h exhibited acceptable range of shear stress along channel wall the actual flowrate used on the micro-perfused platform is much lower. Hence, we can assume that a flowrate of 0.12 ml/h will fall within the safe limits.

Effect of porosity on shear stress:

- Channel width: 800 μm
- Volumetric flowrate: 360 ml/h
- Chamber porosity: 85%; Drag Coefficient A – $1.8\text{e}+11 \text{ 1/m}^2$; Drag Coefficient B – $1.8\text{e}+05 \text{ 1/m}$

Scaffold of various porosity such as 20, 40 and 85% has been simulated. The results clearly showed that, scaffold with high porosity experienced lower shear stress. This has therefore proven the importance of having a porous 3D scaffold for cell culturing studies. In the CFD scaffold porosity studies, the channel width used was set at 800 μm and volume flow rate at 360ml/hr. The reason why we are using such a high flowrate is to reduce the simulation hours. Nevertheless, the effect of porous scaffold has been demonstrated in this study and by lowering the flow rate it will definitely reduce the shear stress experienced by the cells in the cell culturing chamber.

Flow profile and velocity of optimized parameters:

The flow profile of the final optimized parameter (e.g. 800 μm channel width and volumetric flowrate of 18 ml/h) is presented as follow. The first figure demonstrate a 50 % chamber filled while the second figure demonstrate a 100 % chamber filled. From these figure it can be seen that the flow in the chamber where the cells reside is generally laminar although there were small amount of turbulence during the initial perfusion process. Nevertheless, from these simulation results it can be seen that the micro-perfused platform can be operated at these safe perfusion parameters where the cells will experience the least shear stresses.

Figure C1 CFD velocity and flow profile of optimized condition (e.g. 800 μm channel width and volumetric flowrate of 18 ml/h) at 50 % chamber filled

Figure C2 CFD velocity and flow profile of optimized condition (e.g. 800 μm channel width and volumetric flowrate of 18 ml/h) at 100 % chamber filled

Figure C3 CFD velocity and flow profile at 800 μm channel width and volumetric flowrate of 180 ml/h at 50 % chamber filled

Figure C4 CFD velocity and flow profile at 800 μm channel width and volumetric flowrate of 180 ml/h at 100 % chamber filled

Figure C5 CFD velocity and flow profile at 800 μm channel width and volumetric flowrate of 360 ml/h at 50 % chamber filled

Figure C6 CFD velocity and flow profile at 800 μm channel width and volumetric flowrate of 360 ml/h at 100 % chamber filled