Investigation of perfusion culture for developing 3D constructs in tissue engineering

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Investigation of perfusion culture for developing 3D constructs in tissue engineering

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LIST OF NOMENCLATURES

P —fluid pressure

K_d —hydraulic permeability of the constructs

L — radius of construct

H —thickness of construct

x, y —coordinates along the radial and axial directions of the construct

β — the matrix phase of construct

α — the void phase of construct

ε —porosity of construct

V —the averaging volume

l_α, l_β — characteristic dimensions for α and β phases in the averaging volume V

r —the characteristic dimension of the representative elementary volume V

$\sigma_\alpha, \sigma_\beta$ — the respective volume fractions of α and β phases in V

C_β, C_α —oxygen concentrations in the β and α phases in the averaging volume V

$\sigma_{\beta 0}$ —the initial volume fraction of β phase

D_β, D_α —the diffusion coefficient of dissolved oxygen in β and α phases

t —time

R —cellular metabolic rate

K_m —Michaelis constant

R_m —the maximum rate of oxygen consumption for seeded cells

ρ_s —cell seeding density

R_m' —maximum oxygen uptake rate by single cells

U —the local flow velocity

K_{eq} —the equilibrium coefficient of mass at phase interface

D_{eff} —the overall effective diffusivity for V

K —the averaged specific growth rate

R_g —the maximum specific growth rate

K_c —the Contois saturation constant

R_d —the cell death rate

ρ_{cell} —the specific mass density of cell

τ —the local shear stress experienced by the cell

a, b —arbitrary constants

μ —the viscosity of the perfusion medium

u, v —the flow rates along x and y directions respectively

ρ —the density of medium

K_p —a reference value

U_0 —the flow rate along construct central axial axis

n —the unit vector normal to boundary

c_0 —dissolved oxygen concentration in fresh medium

v_c —specific cell volume

t' —dimensionless time

x', y' —dimensionless coordinates along the radial and axial directions of the construct

C_β' —the dimensionless oxygen concentration in the β in the averaging volume V

U' —the dimensionless local flow velocity

u', v' —the dimensionless flow velocity along x' and y' direction

P' —the dimensionless flow pressure

τ' —the dimensionless local shear stress experienced by the cell

$\overline{\sigma_\beta}$ —the averaged volume fraction of cellular mass in the construct

$\overline{C'_\alpha}$ —the averaged DO concentration in the construct

$U_{(r)}$ —flow profile at the inlet

U_{max} —the flow rate along the central axial axis of the inlet,

r_{in} —the distance from the central axial axis

R_{in} —the inlet radius

d —the diameter of cylindrical pores in scaffolds

Q —the volume flow rate in the construct

LIST OF ABBREVIATIONS

3D: three-dimensional

α -SMA: α -smooth muscle actin

AFM: atomic force microscopy

CFD: computational fluid dynamics

DMEM: Dulbecco's modified eagle's medium

DO: dissolved oxygen

ECM: extracellular matrix

EDTA: ethylenediaminetetraacetic acid

FBS: fetal bovine serum

GAG: glycosaminoglycan

HDF: human dermal fibroblast

HUCC: human umbilical cord cell

HVUCC: human vascular umbilical cord cell

IF: interstitial flow

LCD: liquid crystal display

MCLF: medial collateral ligament fibroblast

MMP-1: matrix metalloproteinase-1

MTT: 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

PBS: phosphate buffered saline

PVLF: porcine aortic valve leaflet cell

RGD: Arginine-Glycine-Aspartic acid

SDS: sodium dodecyl sulfate

SEM: scanning electron microscope

TGF- β 1: transforming growth factor beta one

ABSTRACT

Tissue engineering provides a novel method for treating tissue injury and loss. It utilizes artificial bioconstructs grown from patients' own cells to restore or enhance the function of the diseased and lost tissues. The development of three-dimensional (3D) bioconstructs *in vitro* however is challenged by the inefficient mass exchange in the inner construct regions.

One option to solve the problem is perfusion culture, which enforces medium infiltration into constructs and thus improves the mass exchange in inner construct regions. This culture technique not only provides an improved growth environment for 3D construct development, but also offers a convenient way for investigating the cell growth under flow stimulation. This research is thus motivated to explore perfusion culture as an advanced culture technique for 3D construct engineering.

Flow field is the fundamental factor regulating cell and construct growth in perfusion culture. This work firstly has investigated its characteristics in relation to several common bioreactor designs. The modeling results have shown that the conical inlet design is superior to cylindrical inlet design for delivering homogeneous flow in the culture space. With the cylindrical inlet design, a flow zone between the inlet and the scaffold is necessary in order to achieve even flow spreading over the construct surface. To ensure homogeneous flow in the construct region, flow channeling should be avoided.

The work has also modeled the cell growth kinetics in the perfused constructs. While flow changes both the biophysical and biochemical cues around cells in the constructs, the model considers both aspects for cell growth. The simulation has shown that perfusion can greatly improve cell growth in 3D constructs, even at very

small flow rates. Larger flow rates can induce higher cell number growth due to the improved nutrient level and flow shear stress. Consideration of flow shear stress predicts a higher cell number growth in the construct compared to that without shear consideration.

A novel perfusion bioreactor system has been developed for 3D construct development based on modeling results. It satisfies the criteria of good biocompatibility, easy sterilization, direct visual inspection for culture and simple loading and harvest of bioconstructs. The simulated flow mechanics confirms that the flow field in the bioreactor is suitable for 3D construct culture.

Finally, *in vitro* experiments have been carried out with the developed perfusion bioreactor system. Two important culture aspects, the cell retention and cell growth, have been examined in perfused 3D constructs. In the given experimental setup, cell retention in the 3D constructs was dependent on flow rate and flow direction. Cell growth benefited from perfusion culture and was reversely linked to perfusion rates.

The knowledge obtained from this research helps tissue engineers to further understand and utilize perfusion culture for 3D construct engineering.

1 CHAPTER ONE—INTRODUCTION

1.1 BACKGROUND

Loss and injury of tissues due to accidents, diseases and aging can cause severe health problems and heavily impair the quality of people's life [1-3]. Common therapeutic solutions to the problem include autograft (e.g., bone transplantation), allograft (e.g., heart and liver transplantation), electro-mechanical contrivances (e.g., metallic/ceramic implants, artificial heart and pacemaker) and dialysis machines (e.g., liver dialysis). However, these solutions are usually concerned with significant challenges such as the shortage of donor tissues, immunological rejection, device wear and tear and problematic integration with the host. An alternative that can effectively overcome the above mentioned limitations is tissue engineering, which utilizes bioconstructs grown from patients' own cells to help restore or enhance the function of diseased or injured tissues [4-5].

The main tissue engineering approaches can be classified into three categories: cell-only, encapsulation system only, and bioconstructs involving both cells and encapsulation systems [6]. The last category has attracted the most research interests as it allows easy manipulation for the structures and properties of the bioconstructs, improving cell viability and implant integration with the host [7-8].

Typically, to prepare bioconstructs for implantation, autologous cells that are specific for the implantation site are firstly harvested and expanded *in vitro*. Cells are then seeded into three-dimensional (3D) scaffolds, which are designed with suitable mechanical and chemical properties to induce cell growth. The cell seeded scaffolds are afterwards coaxed to assemble into 3D bioconstructs in a dedicated *in vitro* culture environment that mimics *in vivo* conditions. Finally the developed 3D

bioconstructs are implanted into the patient to integrate with the host. The general process flow of the approach is summarized in Figure 1-1.

Figure 1-1: Process flow of tissue engineering approach involving cells and encapsulation systems

1.2 MOTIVATIONS

For successful clinical applications, 3D bioconstructs should possess structural and mechanical properties close to that of native tissues, be biocompatible and have suitable sizes [9]. As cell growth is greatly regulated by external stimulus [10], a proper design of the *in vitro* culture environment is essential for bioconstructs to develop desired properties.

In common tissue engineering practice, bioconstructs are developed through static culture, in which cell-seeded scaffolds are cultivated in stagnant culture medium. A critical problem with this type of culture, however, is that flow penetration into the inner region of the construct is highly hampered by materials at the construct outer layers. Nutrient supply to inner construct region is usually insufficient to meet local cell needs and cell metabolic wastes from the inner construct region cannot be removed in time [11]. As a result, an inferior cell growth environment is induced at the inner region of the construct, impairing local cell function or even causing cell death. The problem becomes increasingly severe with construct size.

An alternative that overcomes the limitation of static culture is dynamic culture, which circulates flow around or through the scaffolds. Dynamic culture supports

both flow diffusion and convection in the system, enhancing flow penetration and chemo-transport in the 3D constructs. It induces more even solute distribution and improves the overall biochemical environment in the inner construct region [12-13]. Dynamic culture hence represents a relatively more advanced culture technique for 3D construct development.

Dynamic culture can be carried out in various bioreactor systems including spinner flask [14], rotational wall vessel [14], hollow fiber bioreactor [15] and perfusion bioreactor [16]. Among them, perfusion culture is relatively more advantageous for delivering medium flow to the internal construct regions. In addition, it is also very flexible for investigating the cell—flow relationship, helping understand cell growth kinetics in 3D environment at the fundamental level [17-18]. Thus, perfusion culture has high potential and important applications in tissue engineering. While the existing studies on the culture technique are still quite limited, this work is motivated to further investigate perfusion culture for 3D construct development in tissue engineering.

Three main elements for the utilization of perfusion culture are the system setup, the culture process and the culture outcome as depicted in Figure 1-2. Important factors involved in system setup include bioreactor design (e.g., shape of the chamber, positioning of the constructs and the design of the flow inlet/outlet), flow input, scaffold design (e.g., channel design, pore size) and cell phenotype. Important aspects related to culture process are the biochemical (e.g., nutrient concentration and distribution) and biophysical (e.g., flow shear, flow pressure) properties of the flow. Important aspects for culture outcome are the cell function (e.g., proliferation, protein deposition, nutrient uptake) and construct assembly (e.g., size increment, shape evolution).

- ① System setup directly affects the culture process biochemically and biophysically
- ② The culture process determines culture outcome; on the other hand , the cell activities shape the culture environment

Figure 1-2: Important factors for *in vitro* 3D construct development under dynamic culture

These three elements are closely inter-related. Firstly, system setup directly determines the flow field in the culture space. The link between system setup and culture process is denoted as ① in Figure 1-2. It would be looked into and the relationship between flow field in the culture and the system design will be established in this work.

Secondly, in dynamic culture, the biochemical and biophysical cues in the culture process have direct and important implications on cell functions. Reversely, cells could also change the flow profile as well as the biochemical environment through activities such as metabolism, secretion and proliferation [18]. The bilateral interaction between culture process and culture outcome is denoted as ② in Figure 1-2. It would be studied in this work so as to better understand the cell growth

kinetics in a given perfusion culture environment.

To summarize, perfusion culture is capable of changing the biochemical and biophysical environment for construct cultivation. It represents an advanced culture technique for *in vitro* 3D bioconstruct development. To better utilize the technique, the flow field in the perfusion bioreactor system and the cell growth kinetics under given perfusion culture environments will be further explored. The study would bring in new insights for the design and utilization of perfusion culture for *in vitro* 3D tissue engineering.

1.3 OBJECTIVES

The work aims to investigate perfusion culture as an advanced dynamic culture technique for *in vitro* 3D bioconstruct development. The detailed objectives of the work are as follows:

- 1) To model the flow field in perfusion bioreactor systems and determine the system designs that can generate homogeneous flow fields for 3D construct culture.
- 2) To model the cell growth kinetics under perfusion culture so as to understand perfusion supported cell growth in 3D construct development at the fundamental level.
- 3) To design and construct a novel perfusion bioreactor with controlled flow field for 3D construct development.
- 4) To investigate the 3D construct development under different perfusion conditions through *in vitro* experiments.

1.4 SCOPE

The detailed scope of the work is as follows:

- 1) To mathematically examine the relationship between the induced flow fields and perfusion bioreactor designs and identify the suitable design features for homogeneous construct culture.
- 2) To develop mathematical models and simulate the cell growth kinetics in 3D constructs under perfusion culture. The models will consider the aspects of flow dynamics, nutrient, cell mass and flow shear in the culture environment.
- 3) To design and construct a direct perfusion bioreactor based on mathematical modeling results from 1) for *in vitro* development of 3D constructs.
- 4) To characterize the flow field in the designed bioreactor and verify its suitability for cell growth and construct development.
- 5) To culture 3D constructs in the fabricated perfusion bioreactor and examine the culture outcome from the aspects of cell retention, cell growth rate and cell number.

The work focuses on the engineering side of 3D tissue development. It does not involve the investigation of cell biology at the intracellular level.

1.5 ORGANIZATION OF THE REPORT

The content organization of the report is as follows:

Chapter 1 introduces the background of tissue engineering and the rational of investigating perfusion culture for 3D construct development. The objectives and scope of the work are stated.

Chapter 2 reviews the literature related to the work from five main aspects: tissue

engineering, dynamic culture and its working principle, effects of dynamic culture on cell growth, different dynamic culture approaches with a focus on perfusion bioreactor and finally mathematical analysis for dynamic culture.

Flow field in dynamic culture is essential in regulating construct development. Its characteristics are largely determined by the bioreactor systems. Chapter 3 presents the mathematical work that investigates the relationship between the induced flow field and perfusion bioreactor designs. Chapter 4 further models the cell growth kinetics in 3D constructs under perfusion culture, considering both effects of flow induced shear stress and nutrient redistribution.

Chapter 5 describes the development of a novel perfusion bioreactor for 3D bioconstruct development. The design and fabrication of the device and the induced flow field are presented.

Chapter 6 reports experimental work carried out with the fabricated perfusion bioreactor for 3D construct development. Two culture aspects, cell retention and proliferation, are examined and discussed in detail.

Chapter 7 concludes the work, suggests future work for this research and lists the publications generated from the research work.

2 CHAPTER TWO—LITERATURE REVIEW

This chapter reviews the current literature related to the work with a focus on tissue engineering and perfusion culture.

Section 2.1 discusses the approach and challenges of tissue engineering. Dynamic culture is pointed out as a solution to the challenges.

Section 2.2 explains why dynamic culture can affect cell growth and tissue development at the molecular level. The important roles of interstitial flow are explicated. The structural connectivity of cells and the mechanotransduction process are discussed.

Section 2.3 elaborates the regulated cellular performance by dynamic culture at both intracellular and cellular levels. Fibroblasts are used as the cell model.

Section 2.4 elucidates different bioreactor systems for carrying out dynamic culture. The general design requirement for the culture system is discussed with a focus on perfusion bioreactors.

Section 2.5 reviews the numerical modeling studies on dynamic culture.

Section 2.6 summarizes the whole chapter.

2.1 TISSUE ENGINEERING

2.1.1 *THE APPROACH OF TISSUE ENGINEERING*

Injury and loss of tissues impose great threats to people's health and life. As an example, in the United States, coronary heart disease caused about 1 of every 5 deaths in 2005 [19]; arthritis and arthritis-attributable activity limitation affect an estimated 21 million of adults and half of those 65 years or older [20].

Common treatments to the problem at present include autograft (e.g., bone transplantation), allograft (e.g., heart and liver transplantation), electro-mechanical contrivances (e.g., metallic/ceramic implants, artificial heart and pacemaker) and dialysis machine (e.g., liver dialysis). These methods however are only temporary solutions and concerned with significant challenges such as shortage of donor tissue and immune rejection. In comparison, tissue engineering grows tissue substitutes from patients' own cells and utilizes the biosubstitutes to restore or enhance the functions of injured or lost tissues [4]. It represents a novel physiological method that overcomes the limitations of above mentioned treatments.

The dominant approach of tissue engineering is based on autologous cells and biodegradable scaffolds as schematically shown in Figure 2-1. In this approach, small amounts of cells are taken from the patient body, expanded and seeded into biodegradable, three-dimensional (3D) scaffolds *in vitro*. The seeded scaffolds are then provided with a dedicated culture environment and induced for cell growth and bioconstruct assembly. Once the bioconstructs are "mature" enough with desired structures and properties, they are implanted into the patient to integrate with the body [4]. As the bioconstructs are developed from patients' own cells, the risk of immune rejection of the implants is lowered and good integration of the implants with the recipient is possible.

Figure 2-1: The dominant approach of tissue engineering [4]

To date, researchers have tried to engineer various types of human tissues. Some engineered products such as skin and cartilage are already commercially available, while tissues with more complex structures are still under intense investigation. The commercial TE products of different development stages at present are given in Appendix A.

2.1.2 THE CHALLENGES OF 3D TISSUE ENGINEERING

Engineering of 3D constructs *in vitro* faces several severe challenges today.

Firstly, mammalian cells are very sensitive to and their functions such as migration, proliferation and adhesion can be largely regulated by the surrounding cues, such as shear stresses, chemical molecules, temperature and cell-cell contact [21-22]. Optimization of the environmental cues in the culture is thus highly important for

the success of 3D tissue engineering. This needs a thorough understanding of the cell-environment interaction at the cellular level, which however is still inadequate at present [23] and should be further explored. One convenient tool assisting the exploration is dynamic culture, in which constructs are cultivated in a dynamic fluidic environment and the cell behaviors under dedicated flow stimulation can be readily studied.

Another challenge with engineering 3D constructs relates to the inadequate mass exchange in the inner construct region. In the commonly utilized static culture, bioconstructs are cultivated in stagnant medium and the mass exchange between 3D constructs and the surrounding medium solely depends on diffusion. Due to the blockage of the scaffold and cellular material at the outer layers of the bioconstructs, nutrients are depleted without adequate replenishment and metabolites are gradually accumulated at the inner construct region. This results in solute concentration gradients across the construct and an inferior growth environment at the inner construct region. It eventually leads to inhomogeneous construct development and limits the size of the bioconstructs, which are highly undesirable. To facilitate better 3D construct development, the mass exchange in the construct should be improved with more advanced culture techniques such as dynamic culture.

2.2 THE MECHANISMS OF DYNAMIC CULTURE

In dynamic culture, culture medium is driven through or over the cultured constructs, generating interstitial flow (IF) in the construct as shown in Figure 2-2.

Figure 2-2: Interstitial flow in the constructs under dynamic culture

2.2.1 INTERSTITIAL FLOW (IF)

The IF generated in dynamic culture helps bring nutrients into and metabolic wastes away from the construct [24]. By drawing medium closer to cells at the inner construct region, IF effectively improves the nutrient availability for cells and assists mass exchange between cells and the culture. IF also carries and redistributes various signaling molecules such as cytokines, growth factors and ions, which can be highly influential for cell behaviors such as proliferation, migration and apoptosis. For example, IF has been found related to the transport of endogenous matrix metalloproteinases (MMPs) which regulate lymphatic migration [25]. Thus, overall, IF facilitates a more homogeneous and improved biochemical environment across 3D constructs, helping even construct development.

IF can also influence cell growth and construct development through flow induced forces. As depicted in Figure 2-3, IF forces can act on cells directly or indirectly through extracellular matrix (ECM) components which are in contact with cells [26]. In particular, the fluid shear stress has been found to be a very important stimuli regulating cell migration [27-28], differentiation [29] and secretion [30].

Figure 2-3: Effect of interstitial flow on cells and constructs ([26])

While the influence of IF on cells and constructs through the biochemical environment could be easy to understand, the influence of IF from the biophysical aspect is not that straightforward. Section 2.2.2 thus focuses on explaining how cells in 3D construct sense the physical signals of IF and transform the signals into particular biochemical reactions.

2.2.2 PUTATIVE MECHANISMS FOR CELL RESPONSE TO PHYSICAL CUES

The process by which cells convert physical cues into intracellular biochemical reactions is known as mechanotransduction [31]. It governs various cell behaviors such as migration and secretion under physical stimulation [32-33]. To understand the mechanotransduction process, firstly the structural connectivity of cells should be recognized.

2.2.2.1 Structural connectivity of cells

It was suggested 25 years ago by Ingber and Jamieson that cells possess a tensegrity architecture [34]. Based on this architecture, the cell is composed of an interconnected network of tensed cables and membranes and compressed struts and substrate anchors. The cell nuclear resides at the center of the network and has an independent integrated network structure. The outer and inner nuclear networks are mechanically connected through elastic filaments [34-35]. Cells are thus hard-wired. Forces acting at a local point of the cell can propagate through the physically coupled structure and result in deformation of the entire cell structure. Figure 2-4 depicts the tensegrity architecture of the cell and the deformed cellular structure under strain.

Figure 2-4: (Left) the tensegrity model of a nucleated cell and (right) deformed cell structure under basal tension strain [31]. The cell is composed of a network of sticks and elastic strings with a separate independent nuclear network in the center. In the deformed cell, the outer and inner nuclear networks are deformed.

In the past years, the mechanical properties of the cell structure have been evaluated by engineering methods such as microcontact printing, magnetic twisting cytometry, optical tweezers and magnetic pulling cytometry [31]. Today the tensegrity model is

widely accepted and the connectivity between different cellular elements is better understood.

Take fibroblasts for example. Fibroblasts attach to ECM through focal complexes which contain integrins as the main transmembrane protein. The extracellular domains of the integrins recognize and bind to various ECM proteins via their Arginine-Glycine-Aspartic acid (RGD) peptide motifs. The cytoplasmic tails of the integrins interact with adaptor proteins (e.g., talin, vinculin, α -actinin, paxilin), which are coupled with cytoskeletal networks such as actin, cytokeratin and tubulin in the cell cytoplasm. The cytoskeletal networks are linked to nesprins, which span the outer nuclear membrane. The nesprins are in contact with inner nuclear membrane proteins such as sun1 and sun2 which bind to nuclear lamins [36]. A pictorial illustration for the complex network of fibroblast is presented in Figure 2-5.

Figure 2-5: Connecting cellular elements [36]

2.2.2.2 Mechanotransduction under dynamic culture

Based on the tensegrity model, cells can sense forces both acting on the cell membrane and on ECM components far away [37]. Their mechanotransduction processes can be stimulated by IF induced flow field in three different ways in general.

First, the contact between the flow and the cell directly strains cell membrane proteins, which could lead to conformational changes of the proteins and regulate the protein binding activities. For example, mechanical strain has activated the high affinity state of integrin $\alpha V\beta 3$ through phosphoinositol 3-kinase dependent conformational change, augmenting the integrin binding to ECM [38]. Mechanical forces at adherent junctions have also activated membrane calcium channels in fibroblasts, generating robust calcium influx and promoting actin polymerization [39].

Secondly, when forces are transmitted along the cellular network, the cytoskeleton components involved in the force transmission path can be physically stretched. Such conformational changes can cause the global reorientation of the network components and exposure of particular protein binding sites. For example, stresses at the cell membrane have led to stiffening response of cells through reorientation of different cellular skeleton components [40] and physical stretch has exposed the cryptic binding sites of talin rods for vinculin binding [41].

Thirdly, the fluid shear stress can elevate cell membrane fluidity [42-43]. This can lead to adaptive cell shape change, activating membrane-associated signaling proteins such as heterotrimeric G proteins [44-46]. It is however notable that cell

shape deformation induced cellular response can be plausibly regarded as being due to the first two mechanisms or, alternatively, to chemical cues [47].

2.3 THE REGULATED CELL ACTIVITIES IN 3D CONSTRUCTS UNDER DYNAMIC CULTURE

2.3.1 IF MANIPULATION FOR CELL ACTIVITIES

The cell response to dynamic culture in 3D constructs involves the synergistic regulation of IF stimulation and the innate cell properties. IF represents the external regulator that enhances solute exchange and applies physical stimulations to the cells. The innate cell properties represent the internal control and can be affected by IF stimulation. Figure 2-6 illustrates the interaction between these two role players and how they work together to induce the cell responses at intracellular and cellular levels.

Figure 2-6: IF manipulation for cell activities [48]

Table 2-1 summarizes the regulated fibroblast activities in 3D constructs under dynamic culture. As shown in the table, various aspects of cells including ECM assembly, autocrine/paracrine secretion, proliferation, alignment, and adhesion and spreading can be affected. Detailed discussions on individual regulated cell activities at intracellular and cellular levels are given in Sections 2.3.2 and 2.3.3.

Table 2-1: Regulated fibroblast activities in 3D constructs under dynamic culture

Cell responses	Cell type	Flow	Major findings	References
ECM component secretion	HDFs	Spinner flask (13 rpm)	Significantly improved collagen (hydroxyproline content) and glycosaminoglycan (GAG) deposition	[49]
	HUCCs	300 to 500 ml/min	Increased collagen, fibronectin and actin formation	[50]
	PVLFs	Pulsatile (10-22 l/min, 5-10 Hz, 1-2 dyn/cm ²) or constant (1-22 dyn/cm ²)	Maintained leaflet synthetic activity near normal levels	[51]
Autocrine /paracrine signaling	HDFs	0.1-0.3 dyn/cm ²	Induced TGF- β 1 and α -SMA expression	[52]
Proliferation	HDFs	0.1 ml/h	Improved proliferation	[53]
	HDFs	Spinner flask (40 rpm)	Improved proliferation	[49]
	HDFs	0.05 ml/min, 0.5 Hz	Further improved proliferation with hydraulic pressure	[54]
Differentiation	HDFs	0.15-0.33 dyne/cm ²	Induced differentiation in autocrine fashion	[52]
Alignment	HDFs	0.1-0.3 dyn/cm ²	Induced perpendicular cell alignment to fluid flow direction	[55]
Adhesion and spreading	MCLFs	10-30 μ L/min	Increased number of intercellular connects and cellular extensions	[56]
Abbreviations: HDF, human dermal fibroblast; HUCC, human umbilical cord cell; PVLF, porcine aortic valve leaflet cell; MCLF, medial collateral ligament fibroblast				

2.3.2 AT INTRACELLULAR LEVEL

2.3.2.1 Extracellular matrix component secretion

Fibroblasts subjected to fluid flow have been observed with incongruous secretion of ECM proteins. In one study of spinner flask culture, human dermal fibroblasts (HDFs) in 3D scaffolds significantly improved collagen (hydroxyproline content) and glycosaminoglycan (GAG) deposition as compared to static culture [49]. In another study with pulsatile flow (300-500 ml/min, 5-15 mm Hg), human vascular umbilical

cord cells (HVUCCs) increased collagen, fibronectin and actin production [50]. In contrast, in the study utilizing pulsatile (10-22 l/min, 5-10 Hz, 1-2 dyn/cm²) flow, heart valve leaflet fibroblasts in 3D only maintained the protein and GAG synthesis at basal levels without synthesis improvement [51]. The dramatic difference in culture results might be linked to the variances of culture system setup, such as cell models, scaffold properties, the flow profiles, as well as the circulation of cell secreted autocrine factors.

2.3.2.2 Autocrine/paracrine factor secretion

Fibroblasts release autocrine/paracrine factors to help modulate the activities of adjacent cells, such as epidermis division and differentiation and vascular smooth muscle cell proliferation [57]. IF with shear stress of 0.1-0.3 dyn/cm² induced transforming growth factor beta one (TGF- β 1) and α -smooth muscle actin (α -SMA) expression in HDFs in 3D [52].

2.3.3 AT CELLULAR LEVEL

2.3.3.1 Proliferation

Dynamic flow culture can promote cell proliferation at low flow rates. For example, perfusion culture at 0.1 ml/h [53] and spinner flask culture at 40 rpm improved the proliferation of HDFs in 3D [49]. When cyclic hydrostatic pressure was added in perfusion culture, further improved cell proliferation of human foreskin fibroblasts in 3D was resulted as compared to that in perfusion only environment. The growth improvement was attributed to the nutrient penetration in tissue constructs enhanced by the hydrostatic pressure [54].

2.3.3.2 Differentiation

Fluid flow with shear stress ranging between 0.15 and 0.33 dyne/cm² induced

fibroblast-to-myofibroblast transdifferentiation in 3D in a $\alpha1\beta1$ integrin dependent manner [52]. This cellular event occurred in the absence of exogenous mediators, indicating that fluid flow alone was able to induce fibroblast differentiation. The flow also promoted the production of TGF- $\beta1$, which was a typical growth factor promoting fibroblast-to-myofibroblast transdifferentiation. Application of TGF- $\beta1$ blocking antibodies eliminated the transdifferentiation. Thus the cell-derived TGF- $\beta1$ under fluid flow might be the reason for the flow induced fibroblast-to-myofibroblast differentiation [52].

2.3.3.3 Alignment

Under shear flow (0.1-0.3 dyn/cm²), fibroblasts in 3D collagen scaffold aligned perpendicular to fluid flow [55]. Given that even acellular collagen matrices aligned weakly upon exposure to flow, it was possible that flow first induced collagen fiber alignment, which provided contact guidance for the cells to orient along the aligned matrix; later, the oriented cells further remodeled and aligned their surrounding matrix fibers [58]. This inference was supported by the time-lapse atomic force microscopy (AFM) observation that during cell alignment, cells reoriented individual collagen fibers and deformed the matrix [59].

2.3.3.4 Adhesion and spreading

Cell adhesion and spreading on ECM proteins or substrates play a critical role in shaping cellular morphology and tissue structure assembly [60]. Flow shear stress could promote filopodial network development and spreading of fibroblasts [56]. As cell adhesion is mediated mainly by integrins and the intracellular signals of the cell, it is likely that shear stress influences cell adhesion through modifying the related integrins.

2.4 THE METHODS OF DYNAMIC CULTURE

Dynamic culture is carried out in bioreactor systems, which allocate cells/constructs in a dedicated culture environment with strictly controlled biochemical (e.g., nutrients, signaling molecules) and biophysical (e.g., shear stress, hydrostatic pressure) cues [61-62]. Normally, constructs pre-cultured in such systems have more stable cell functions and integrate better with host tissues after implantation compared to those without bioreactor culture experience [63-64].

2.4.1 *DIFFERENT BIOREACTOR SYSTEMS*

The concept of bioreactors was initiated from fermentation and protein production industry [65]. Some of these bioreactor designs, such as fluidized bioreactor, packed bed bioreactor [66], stirred tank [67] and hollow fiber reactor [68], were then modified for mammalian cell culture and tissue development with the emergence of tissue engineering. Later, novel bioreactors specific for mammalian cell growth have also been developed, including such as perfusion bioreactors [69-70] and rotating wall vessels [71-72]. Figure 2-7 presents the schematic drawings for the six commonly used dynamic culture bioreactors in 3D tissue engineering today.

Figure 2-7: Schematic drawings of six commonly used dynamic culture bioreactors in 3D tissue engineering

Among the different designs, mixed bioreactors (Figure 2-7 a) agitate seeded constructs in culture flask or dishes with an orbital shaker or gyrator [73].

Spinner flask mixes the culture medium around seeded constructs with an inner blade or magnetic stir bar. For suspension cells, cell aggregates are freely suspended in the medium (Figure 2-7 b1). For adherent cells, constructs are attached to needles which extend from the flask cap into the inner flask space (Figure 2-7 b2) [74].

Rotating wall vessel (RWV) (Figure 2-7 c) rotates the vessel horizontally during culture. The scaffolds are suspended in the medium due to balanced fluid drag force and the gravitational force [75-76].

Fluidized bed bioreactor (Figure 2-7 d1) drives flow through the bed of cell carriers from the bottom of the chamber. The carrier bed is kept in a stable state as long as the flow velocity is constant [66]. Fixed bed bioreactor (Figure 2-7 d2) has a design similar to fluidized bed bioreactors but with an additional sieve at the flow inlet to reduce the flow turbulence [66].

Hollow fiber bioreactor (Figure 2-7 e) cultures cells in the intracapillary space of fiber bundles. Oxygen and medium inside the capillary diffuse into the intracapillary region through the semi-permeable fiber wall [68, 77].

Perfusion bioreactor (Figure 2-7 f) drives flow through 3D constructs directly. It typically consists of a pump, a medium reservoir, a control valve and a culture vessel where 3D scaffolds reside [78-79].

Every dynamic culture bioreactor has its own advantages and limitations. Mixed flask is easy to set up, however its culture efficiency can be quite low. Instead, it is more often used for cell seeding [73]. Flows in spinner flask and fluidized bed bioreactor are usually turbulent, which can result in good mass exchange and low solute concentration gradient in the medium. However they also impose high shear stress on cells which may cause cell damage [74, 80]. Fixed bed bioreactor introduces low shear stress to cells but is concerned with solute concentration gradient in the flow direction [66]. Flow in rotating wall vessel is usually laminar with good mass exchange. It however may face unstable construct movement and collision between the construct and vessel wall [71, 81-82]. Hollow fiber bioreactor delivers laminar flow in the lumen, but its design greatly limits the tissue size [77, 83]. The flow characteristics, strengths, weaknesses and common applications of different dynamic culture bioreactors are compared in Table 2-2.

Table 2-2: Comparison between different dynamic culture bioreactors

Bioreactors	Common Applications	Flow characteristics	Strengths	Weaknesses	References
Mixed flask/plate	More often for construct seeding rather than tissue growth	Possibly very low turbulence and shear strain depending on the gyration rate	Inexpensive; easy to operate	Surface aeration; slow oxygen transfer; weak cell function and thin construct	[73]
Spinner flask	Wide application for culture of various tissues and suspension cells	Turbulent, uneven and periodic flow with the stirrer rotation (commonly at 50 rpm); high shear stress	Homogeneous solution mixing; highly enhanced mass transfer; improved cell function and proliferation	High shear stress; possible cell detachment and damage; commonly resulting in 0.5 mm thick capsule for cultured scaffolds	[74]
Rotational wall vessel (RWV)	Wide application for culture of liver tissue, cardiac tissue, cartilage and bone; stem cell expansion	Laminar flow; suspended constructs during culture process; partial microgravity in the upper region of the vessel	Enhanced solute exchange between cells and culture medium; low shear stress; homogeneous culture environment	Possible collision with vessel wall for heavier-than-medium particles; microgravity environment; changing construct movement along with tissue development	[71, 81-82]
Fluidized bed	Expansion of hematopoietic cells; bioartificial liver system	Turbulent flow	Homogeneous mixing; high aeration rate; continuous nutrient and oxygen supply; ease of scale-up	High shear stress; possible cell detachment and damage	[66, 80]
Fixed bed		Laminar flow	Low shear stress; continuous nutrient and oxygen supply	Concentration gradient; possible hypoperfusion due to flow channeling; difficulty in scaling up	
Hollow fiber	Bioartificial liver system; culture of endothelial cell, lymphocytes and hepatocytes. Very limited application for 3D tissue culture	Laminar flow in the lumen	High surface to volume ratio; short diffusion distance between cells and perfusing medium	Concentration gradient along flow direction; small space for construct development; high flux immunoprotective membranes required; difficult functional cell attachment on durable materials; difficulty in scaling up and sampling; hypoperfusion	[77, 83]
Perfusion	Very wide application for almost every type of tissue culture	Laminar or turbulent	Continuous nutrient and oxygen supply and metabolite removal; good cell function and improved cell ingrowth; especially useful for culturing scaffolds with inhomogeneous culture needs	Concentration gradient in flow direction; hypoperfusion due to flow channeling	[17, 84-85]

Most of the above mentioned dynamic culture bioreactors can only create fluid eddies in the superficial pores of scaffold. Relatively, perfusion culture, which directly pumps culture media into the 3D cellular constructs, is more efficient in delivering flow into the interior scaffold regions [17]. In addition, perfusion bioreactor is also extremely flexible in delivering customized flow profiles to meet specific culture needs [62, 79, 86-88]. Thus, perfusion culture is selected in this work for investigating dynamic culture for 3D tissue engineering *in vitro*.

2.4.2 PERFUSION CULTURE DESIGN

There exist multiple design factors for perfusion culture as summarized in Table 2-3. These factors fall into two categories with respect to cell activities: Category 1 of external influencing factors, Category 2 of internal influencing factors.

Table 2-3: Influencing factors in dynamic culture

Influencing factors		Aspects in detail
Category 1	Flow	Flow shear stress level Flow rate Flow direction Flow duration Flow frequency Flow variation
	Perfusate	Presence of calcium ions Presence of serum
	Nutrient supply	Dissolved oxygen level and distribution Nutrient gradient
	Signaling molecules	Endogenously applied autocrine/paracrine factors Self-secreted autocrine/paracrine factors
	Scaffold/substrate	Topography (for 2D) Microstructure (for 3D) Porosity (for 3D) Permeability (for 3D) Stiffness Chemical property
Category 2	Cell	Cell types Phenotype of the cells

2.4.2.1 External factors

The first category consists of the biophysical and biochemical cues in the culture environment. It encompasses the external factors of the culture including flow [62, 86-89], the nutrition and signaling molecules and the scaffold property [90]. It can be readily manipulated by engineering methods.

Perfusion flow can be continuous, pulsatile or oscillating. Table 2-4 summarizes the previous methods for obtaining these different flow profiles.

Table 2-4: Previous methods for generating different flow profiles in perfusion culture

Flow Profile	Method	Reference
Continuous	(Peristaltic) pump	[78-79]
Pulsatile	Programmable/pulsatile pump	[91]
	Valve control	[62]
	Air pressurization control	[92-93]
	Diaphragm deformation control	[94]
Oscillating	Reversible pump	[87]
	Flow sensor control	[95]

There are complex interactions between different influencing factors in the culture system and the effects from individual influencing factors could even be conflicting. For example, higher flow rate promotes better nutrient transport and mass exchange between cells and medium, improving cell growth. It however could at the same time induce high levels of shear stress. While mild shear stress can help stimulate physiological cell growth, excessive shear stress might cause detrimental effects on cells [96]. Thus, to gain the most benefits of the culture environment, the effects from individual factors need to be balanced systematically. Suitable nutrition and flow force levels should be both considered when the perfusion flow rate is determined.

2.4.2.2 Internal factors

The second category involves the innate cell phenotype, which is the basis for the bioactivities in the bioconstruct [97].

As different cell types have their own requirements for growth environment, the design of perfusion bioreactor systems should be tailored to cell types accordingly. For example, myocytes are extremely vulnerable to shear stress and hypoxia [98]. To perfuse constructs with myocytes, minimizing flow shear stress is necessary [99] and oxygen carriers for improving the oxygen availability in the culture may be needed [100-102]. In comparison, chondrocytes can survive relatively high levels of shear stress and hypoxia. For the cultivation of chondrocytes, shear stress to cells is preferred and oxygen supply can be kept at a normal to low level [103].

2.4.2.3 Determination of influencing factors

The co-existence of various influencing factors complicates the culture system and makes it very difficult to determine the optimal perfusion culture environment for 3D construct development. As a general guideline, the flow level *in vitro* should be determined with reference to the *in vivo* IF (0.1-4 $\mu\text{m/s}$ flow rate, 0.005-0.015 dyn/cm^2 flow shear stress) [104]. The flow frequency should be designed based on the anatomy part of the human body where cells are taken from [89]. It is also preferable to match the hydraulic conductivity of 3D matrices with that of interstitium *in vivo*, which is in the order of 10^{-8} ($\text{cm}^2/(\text{mm Hg-s})$) for connective tissues [24]. It is worth pointing out that while the best efforts should be made to mimic *in vivo* for *in vitro* culture design, it is unlikely for *in vitro* culture to fully emulate the *in vivo* environment due to the extreme complexity of the *in vivo* condition. Cell behaviors observed *in vitro* thus might be different from that *in vivo*.

2.4.2.4 Design concerns

One concern with perfusion culture is the possibility of “hypoperfusion” in the construct. As illustrated in Figure 2-8, in such a case, rather than passing through the construct matrix evenly, the flow selects paths with the least resistance. The cells at the bypassed regions of the construct then experience “hypoperfusion” with hampered flow influences compared to cells at other regions [105]. To minimize the chances of “hypoperfusion” thus promote even flow stimulation to the construct for homogeneous construct development, scaffolds should be fabricated with homogeneous structure. In addition, to ensure medium passes through the scaffold, bioreactor vessel should be designed with a press fit for scaffolds.

Figure 2-8: Hypoperfusion in 3D constructs

2.4.3 MAIN PERFUSION BIOREACTORS IN LITERATURE

Different perfusion bioreactors have been developed by previous researchers for culturing different cell types in 3D. As bioreactors developed for the same type of cells are more suitable for comparison, this section focuses on perfusion bioreactors that are designed specifically for fibroblast based 3D construct development.

Smith *et al.* designed a flat-bed perfusion bioreactor for culturing fibroblasts in a porcine-derived collagen scaffold [106]. It consisted of two blocks with internal indentation for housing cellular matrix. Direct perfusion to the scaffold was supplied from the ports on two sides of the block. The maximum thickness of scaffold in the

bioreactor was 2 mm, which can be a limitation for studying cell behavior in thicker matrices. The blocks were covered with stainless steel pressure plates which prevented direct visual monitoring of the culture. Flow was directed to the scaffold through two concentrated regions, which did not spread evenly over the scaffold cross section and imposed inhomogeneous stimulations to the scaffold [106].

Ng *et al.* designed a 3D perfusion culture model for fibroblasts embedded in collagen gel [107]. The design sandwiched cell-suspended gel between two glass cover slips and perfused culture medium through the gel radially. The group used this setup to study the IF effects on fibroblast alignment. The design was only suitable for gel material and cannot be applied for solid scaffolds such as collagen sponges and most matrixes made from synthetic materials [107].

Mizuno *et al.* developed a culture system providing both hydrostatic pressure and perfusion to human dermal fibroblasts seeded in a gel/sponge construct [54]. The construct was suspended inside the perfusion chamber. This design generated micro-flows in the surface region of the scaffold, but could not deliver flow into the inner region of the scaffold. In addition, the system did not allow direct visual observation and was not compatible with incubator culture [54].

2.5 NUMERICAL ANALYSIS OF DYNAMIC CULTURE

With current sensing technologies, it is very difficult for tissue engineers to experimentally check the cell growth condition in 3D constructs at the micro-level in real time. Very often, computational fluid dynamics (CFD), which uses numerical methods and algorithms to study the culture, has to be relied on.

2.5.1 ROLES OF CFD IN DYNAMIC CULTURE

CFD can be utilized to analyze the culture condition at micro-level with respect to both flow fields (e.g., shear stress, mass delivery) and cell functions (e.g., migration, proliferation, and metabolism) in given dynamic culture systems. For example, CFD modeling using Lattice-Boltzmann method was utilized to simulate the flow conditions in perfused cylindrical scaffolds. It was found that the shear stress with an average value of 5×10^{-5} Pa had improved cell proliferation and shear stresses of higher values had upregulated expression of bone marker genes [108].

CFD can also be used to evaluate and predict the culture outcome of particular experimental setups (e.g., scaffold geometry, flow rate, flow space). It thus helps optimize the experimental design before device fabrication and actual culture experiments. For example, CFD utilizing modified Contois cell growth kinetics (a model which links cell growth with nutrient saturation and cell number) showed that when the void space in the scaffold was gradually taken up by cell proliferation and deposition, the flow-mediated shear stress increased correspondingly. The maximum shear stress that cells in the construct would experience was then calculated and evaluated to check whether it would induce cell necrosis in the perfusion culture system [17].

2.5.2 LIMITATIONS OF CURRENT CFD STUDIES

CFD models are usually developed based on specific system setups. While dynamic culture involves complex interplay of different influencing factors (e.g., scaffold geometry, scaffold location, cell number, cell type, culture medium, flow input), the developed CFD models may easily become inapplicable for the culture system if any factor in the system is modified. To enable wider application for the modeling

results, studies based on modular designs of culture systems are preferred and needed in the future.

In most CFD models, only nutrient and oxygen availability have been considered as the regulating factors for cell growth and the flow induced forces have been largely ignored [17, 85, 109-111]. However, as discussed in Section 2.2, dynamic culture influences cell and construct growth both biochemically and biophysically. CFD models with consideration of biophysical effects are thus expected to give a more realistic description for the culture process compared to that without such consideration. To further clarify the flow impacts on cell growth for 3D construct development, CFD models that consider the effects of both nutrients and flow force should be developed.

2.6 SUMMARY

Tissue engineering provides a novel physiological method for treating injured or lost tissues with bioconstructs developed from patients' own cells and biodegradable scaffolds. Dynamic culture, which enforces flow penetration into 3D constructs, has been attracting increasing research interest due to its advantages in improving the construct development compared to static culture. Among different dynamic culture methods, perfusion culture is relatively more advantageous in delivering flow into the inner construct region. It is also very flexible in culture design with easy adjustment of design factors such as flow input, culture vessel configuration and scaffolds. It thus has great potential as a helpful and convenient tool for studying 3D construct development in tissue engineering.

3 CHAPTER THREE—NUMERICAL ANALYSIS OF FLOW FIELD IN PERFUSION BIOREACTOR SYSTEMS

In order to take full advantage of perfusion culture for 3D construct development, the flow field in the culture process should be clearly understood. This chapter investigates the perfusion flow fields in relation to several modular features of the culture systems with computational fluid dynamics (CFD) analysis. The elucidated knowledge helps tissue engineers to design perfusion systems that deliver homogeneous flow stimulation for 3D construct development.

3.1 BACKGROUND

Perfusion culture provides a dedicated culture environment for the *in vitro* 3D construct development [112-113]. It enhances flow penetration into the construct, applies physical stimulation (e.g., shear stress, flow pressure) to cells and redistributes biomolecules (e.g., nutrients, metabolic waste) in the culture [114-116]. To make good use of perfusion culture for 3D tissue development, the flow field in the culture process should be studied and well understood.

Computational fluid dynamics (CFD) modeling is one of the more effective techniques for flow field characterization. Most existing CFD studies in tissue engineering have focused on the flow in a given system [79, 117-119]. While such studies do help tissue engineers understand the flow in the system, their referential significance to different system designs is largely limited. To help tissue engineers resolve the flow field in common culture practice, knowledge that spans the boundaries of individual system designs is preferred. For example, it is useful to suggest that in a commonly seen circular shaped perfusion bioreactor, designing the inlet and outlet in a semicircular shape could induce more uniform hydrodynamic

stress and nutrient distribution [120]. This work is thus motivated to investigate the flow field related to several modular features of perfusion system: the flow inlet, the flow region between the inlet/outlet and the construct and the flow channels in the culture.

3.2 MODEL GENERATION

3.2.1 *MODEL DESIGN*

The flow field in perfusion culture can be influenced by many factors such as scaffold structure, bioreactor configuration, cell activity and flow input (e.g., rate, frequency). This work focuses on three aspects in particular: the flow inlet, the flow zone between flow inlet/outlet and construct and the flow channels in and around the scaffold. These aspects are chosen because, first, they are relatively common design modules for perfusion culture, which makes the generalization of the obtained knowledge possible; and secondly they are expected to play important roles on shaping the flow field. It is hypothesized that bioreactor chambers with a conic inlet design, a buffer zone between the flow inlet and the construct and absence of flow channels can deliver a more even flow field for construct development compared to that with a cylindrical inlet design, absence of flow zone between inlet and the construct and presence of flow channels.

3.2.2 *MODEL SETUP*

Study models are developed based on commonly found perfusion systems in practice. In the models, flow enters the bioreactor from the inlet, passes through the scaffold region and exits the bioreactor from the outlet. The bioreactor chamber has a tubular shape concerning that tubular structure is relatively easy to machine. Schematics of the model systems are shown together with the simulation results in

the result section. The scaffolds are set as 5 mm thick porous discs with permeability of 10^{-10} cm^2 [24]. 5 mm is chosen as it is the thickness of the thickest scaffold that has been reported in literature [121]. The radius is arbitrarily chosen as 6.5 mm, which is an uncritical parameter for scaffold fabrication process and thick construct development. The flow rate at the bioreactor inlet is arbitrarily set at 1 ml/min. Perfusion medium is assumed Newtonian with consistent properties during the culture process. The density of the medium is 1.009 g/cm^3 and the viscosity of the medium is $8.4 \times 10^{-3} \text{ g/(cm-s)}$ [85].

3.2.3 SIMULATION

In the models, the perfusion flow fields are symmetrical about the central axial plane of the bioreactor. To minimize computational cost, only the flow field on the symmetry plane is simulated and presented. The study assumes unchanged construct structure during the culture. To calculate the formed flow field in the porous construct region, Brinkman's equation which is commonly used to describe viscous flow in a porous medium [122] is used as presented in Equation (3.1). Equation (3.2) describes the mass conservation for the flow.

$$\nabla P = \mu \nabla^2 U - \mu U / K_d \quad (3.1)$$

$$\nabla U = 0 \quad (3.2)$$

P —pressure in dimension of [Pa or kg m/s^2],

K_d —hydraulic permeability of the constructs in dimension of [m^2],

U —velocity of flow in the matrix in dimension of [m/s],

μ —dynamic viscosity of the fluid in dimension of [Pa-s]

In Equation (3.1), the left side term is the flow pressure difference in the characteristic length; the first term on the right side is the viscous force which is an important component in the construct culture, where the IF is usually very small and the viscous force dominates convection; the second term on the right side is the pressure drop due to flow in the void space of the porous region. The use of the viscous term allows the equation to be used for non-slip boundary conditions such as flow near internal obstructions or bounding walls. Brinkman's equation treats the scaffold interstitium as a porous medium and flow is constrained in the void space of the region. The porous medium is assumed to be continuous [123-124].

If taking the individual fibers into consideration, the medium and flow are discontinuous. At the cellular level, IF induced shear stress is likely sensed by cells with individual molecules or adhesion plaques (see Section 2.2), which would be on the same size scale or smaller than the flow discontinuities. Using a continuum approximation such as Brinkman's equation thus would give averaged descriptions of flow and flow induced cell responses [125]. In comparison, Navier-Stokes equation may give a more precise simulation [125]. However, given the excessive complexity and computational cost of including each fiber and void into simulation, Equation (3.1) is often preferred for studying the flow field in porous matrices [108, 118, 126].

For other flow regions in the culture space where free flow is present, Navier-Stokes equation (Equation (3.3)) which governs non-turbulent, Newtonian fluid is used for describing the flow field.

$$\rho \left(\frac{\partial U}{\partial t} + U \cdot \nabla U \right) = - \nabla P + \mu \nabla^2 U \quad (3.3)$$

ρ —the density of the fluid in dimension of [kg/m³].

The left side of the equation describes the change of flow momentum. The first term on the right side is the pressure gradient and the second term is the viscous force of the flow.

Commercial software FEMLAB 3.1 (COMSOL) is utilized to construct the study models, mesh the structures and solve the CFD Equations (3.1) to (3.3). Mesh sizes are selected based on the criteria that the discrepancy of calculation results is within 4%. To equalize the viscous momentum transport at the interface of the porous scaffolds and free flow regions, viscous stress tensor is used for both Brinkman's and Navier-Stokes's equations.

3.3 SIMULATION RESULTS

3.3.1 THE FLOW INLET EFFECT

Two flow inlet designs are compared: the cylindrical shape and conic shape. Figure 3-1 presents the respective schematics. For both designs, the chamber section between the inlet and outlet has the same length (5 mm) and radius (6.5 mm) as the scaffold. Fluid flushes onto scaffold immediately after it passes the flow inlet. The cylindrical inlet has a radius of 2 mm. The two rims of the conical inlet have radii of 2 mm and 6.5 mm respectively as indicated in the Figure. The length of the inlet region in both designs is 2 mm.

Figure 3-1: Perfusion bioreactor with inlet in (left) cylindrical shape and (right) conic shape. Arrows indicate the flow direction.

Figure 3-2 presents the simulated flow rate profiles on the axial symmetry plane of the bioreactor chamber for the two designs.

Figure 3-2: Flow velocity profiles in the bioreactor chamber when flow inlet has a (left) cylindrical design and (right) conic design. Arrows indicate flow direction.

Figure 3-2 shows the flow profiles in the culture chamber vary significantly with the two different inlet designs. With cylindrical flow inlet, the flow follows its original horizontal direction along its way through the scaffold. As a result, flow concentrates at the central regions of the scaffold without reaching the scaffold periphery. In comparison, when the flow inlet has a conical shape, flow diverges in the inlet following the inlet contour and spreads more evenly over the scaffold surface upon meeting the scaffold.

Figure 3-3 presents the simulated pressure profiles on the axial symmetry plane of the bioreactor chamber for the two designs.

Figure 3-3: Pressure profiles in the bioreactor chamber when flow inlet has a (left) cylindrical design and (right) conic design. Arrows indicate flow direction.

The pressure distribution profile in the scaffold in Figure 3-3 confirms with the flow profile in Figure 3-2 for both inlet designs. With cylindrical inlet, flow pressure is much higher in the scaffold region near the flow inlet than that at other regions of the scaffold. In comparison, when the inlet is conical, the flow pressure remains uniform on the same radial plane of the construct. For both designs, the flow pressure in the construct gradually decreases along the axial direction of the bioreactor due to the change of static pressure. Figures 3-2 and 3-3 show that in the bioreactor with conical inlet design, the scaffold is exposed to more even flow stimulation.

To further explore the conical inlet design for perfusion culture, the impact of its length on the flow field is examined. Figure 3-4 presents the flow rate and pressure distribution profiles in the bioreactor when the conical inlet has a length of 4 mm.

Compared to the condition when the inlet length is 2 mm (right side of Figures 3-2 and 3-3), little differences are observed.

Figure 3-4: (Left) flow rate and (right) pressure profiles in the bioreactor chamber when the conic flow inlet has a length of 4 mm. Arrows indicate flow direction.

3.3.2 THE INTER INLET/OUTLET-CONSTRUCT FLOW ZONE EFFECT

The flow zone between the flow inlet/outlet and the construct is expected to greatly shape the flow profile around the scaffold. Its influence on the flow field in the culture chamber is simulated in Figure 3-5.

Figure 3-5: Flow profiles in the bioreactor chamber when the length of the flow zone between the inlet/outlet and scaffold is: (a) 0.5 mm, (b) 1 mm, (c) 2 mm and (d) 3 mm

Based on Figure 3-5, with a larger flow zone between the inlet/outlet and the construct, flow is more evenly distributed across the outer surfaces of the scaffold. The zone serves as a buffer for fluid coming out of the narrow inlet to diverge and reach an even spread over the scaffold outer surface. Similarly, it allows the flow

coming out of the construct to gradually converge to the outlet. Inside the scaffold, there is little difference in the flow profiles between different designs. It could be due to the low flow permeability of the scaffold, which slows down the infiltrating flow, forces flow to spread over the scaffold upon contact, and evens out the flow in the scaffold region.

Thus, in condition of cylindrical flow inlet, flow passes through the scaffold central regions without spreading over to the construct periphery at the absence of the buffer zone (left of Figure 3-2). In order to obtain a uniform flow profile for even construct development, a buffer zone is necessary between the inlet/outlet and the scaffold. Even when the zone volume is as small as that in Figure 3-5a, such flow zones could help greatly improve the flow distribution.

3.3.3 THE FLOW CHANNEL EFFECT

The diameter of bioreactor chamber can be the same as or larger than that of the construct. Figure 3-6 compares the simulated flow fields for these two cases.

Figure 3-6: Flow fields in the culture space when the diameter of culture chamber is (left) the same as and (right) 0.2 mm larger than that of the scaffold. The latter results in flow channeling at the scaffold periphery.

Simulation demonstrates that when bioreactor chamber and scaffold have the same size, flow is forced through the scaffold evenly (left side of Figure 3-6). In comparison, when bioreactor chamber has a diameter larger than that of the scaffold, external flow channels around the scaffold are induced (right side of Figure 3-6). Flow squeezes through external channels and bypasses the constructs. Thus, size difference between the culture chamber and scaffold should be avoided for even construct perfusion.

The homogeneous flow profile on the left side of Figure 3-6 requires uniform scaffold structure. In some tissue engineering practice, internal flow channels are designed in 3D constructs to help improve mass delivery in the inner construct regions [127-130]. Further studies are thus carried out to determine the influence of the internal flow channels on the flow field in the construct. Three design aspects of the internal channels are examined: the opening position, orientation and size.

Internal flow channels can have very complex structures in the constructs in practice. However, for illustration purpose, the study adopts their simplest form — a tubular structure with both ends on the surfaces of the construct. Based on the notion that external flow channels commonly exist around the scaffolds in actual culture practice, the study model for internal flow channel effect is built upon the design of the right side case in Figure 3-6. While the simulation is conducted for the entire bioreactor region, only the flow field in the construct region is of interest and presented.

3.3.3.1 Channel opening position

Flow fields in scaffolds with different channel opening designs ($\varnothing$ 0.5 mm) are compared in Figure 3-7. In design (a), the internal channel starts from and ends at

the periphery surfaces of the construct. In design (b), the internal channel starts from the same place on construct periphery surface as that in design (a) but ends at the construct top surface.

Figure 3-7: Flow fields in the construct when internal channel ends at the (a) periphery surface and (b) top surface of the construct respectively. Design (b) supports higher flow in the channel region. Arrows indicate the main flow direction.

Simulation shows that the design of the internal channel opening can affect the flow field in the construct. With fluid entering the constructs from the construct top surface, channel opening on the construct top in design (b) allows part of the medium to enter the channel cavity without blockage of the scaffold material. This leads to a higher flow rate in the channel compared to that in design (a), in which the channel with both openings on construct periphery surface is shielded from direct flow entry.

Figure 3-8 plots the flow velocity along the axial direction of the scaffold for the two cases. With channel opening allowing direct flow entry, the maximum flow rate in

the channel reaches above 0.2 cm/s, compared to that of around 0.08 cm/s when channel opening is shielded from direct flow entry. The plot also shows that the flow rate at the construct regions near the channel increases with that in the channel, which can be expected to help improve the local mass exchange and cell growth.

Figure 3-8: Flow velocity plot along the axial direction of the construct (along the red dotted line in Figure 3.7) when channels have different opening designs. Channel opening position that allows direct flow entry supports higher flow rate in the channel and neighboring construct regions.

3.3.3.2 Channel orientation

Flow fields in scaffolds with internal channels ($\varnothing$ 0.5 mm) tilting at different angles are simulated and presented in Figure 3-9. All the channels start from the same location on the construct periphery surface. They end at different locations on the left outer surface of the construct due to different tilting angles.

Figure 3-9: Flow fields in the construct when internal channels are tiled (a) 5° , (b) 7° and (c) 10° from the vertical direction. Arrows indicate the flow direction. The more the channel is tilted towards the incoming flow, the higher the flow rate in the channel.

Based on Figure 3-9, with main flow propagating horizontally from the left outer surface to the right outer surface of the construct, internal flow channels with bigger tilting angles from the vertical direction in the construct are more aligned to the flow. This makes it easier for the flow to enter the channel and results in an increased flow rate in the channel and neighboring construct regions. Figure 3-10 compares the flow velocities in constructs when internal channels are tilted differently.

Figure 3-10: Plot of flow velocity in the constructs (along the red dotted line in Figure 3-9) when internal channels have different tilting angles from vertical direction. The bigger the angle, the more the channel is slanted towards the main flow direction and the higher the flow rate in the channel.

As the channel tilting angle increases from 5° to 10° from the vertical direction, the flow rate in the channel and neighboring construct regions increases gradually. Thus the more the channel is aligned to the main flow stream, the higher the flow rate in the channel is.

3.3.3.3 Channel size

The channel size's effects on the flow field are studied with consistent opening and orientations of the channel. Figure 3-11 presents the corresponding flow fields in the scaffold when the channels have diameters ranging from 0.1 mm to 0.5 mm. Figure 3-12 plots the corresponding flow rates along the construct's axial direction.

Figure 3-11: Flow velocity profiles in the construct when internal channels have different diameters: (a) 0.1 mm, (b) 0.3 mm and (c) 0.5 mm. Arrows indicate the main flow direction.

Figure 3-12: Flow velocity in the construct along the axial direction (along the red dotted line in Figure 3-11) when internal channels have different diameters. The bigger the channel diameter, the higher the flow rate in the channel.

The results indicate a close relationship between the channel size and the flow profile in the construct. The larger the channel size is, the easier the flow enters the channel and the higher the flow rate develops in the channel and neighboring scaffold regions. It is noted that the flow rate increment in the channel is not in proportion with the channel size increment. Rather, with larger channels, the same size increment leads to greater flow rate increase. Such a relationship should be related to the particular channel orientation and opening design in the model.

3.4 DISCUSSION

Perfusion culture is advantageous for improving the culture environment and mass exchange for 3D tissue construct development. This work has numerically analyzed the flow field dependence on perfusion system designs with focus on the design of flow inlets, flow zone between inlet/outlet and scaffold and flow channels.

The diameters of the cylindrical inlet and the smaller rim of the conical inlet in the simulation study are both set as 4 mm. These diameters are determined based on the size of a commercially available tubing adapter that connects the bioreactor with tubing delivering 1 ml/min flow rate. The effort is made so as to make the simulation results easier to be referred to for tissue engineers, who for convenience usually prefer using existing components from the market for constructing bioreactors.

Simulation results have verified our hypothesis that the inlet design of culture chamber can have a heavy influence on the flow profile. The conical inlet could induce a more homogeneous flow profile, thus promoting more even construct development compared to cylindrical inlet. With the conical inlet design, fluid diverged following the contour of the inlet and the length of the inlet region imposed no effect on the eventual flow field on construct surface. The simulation has also

shown that the volume of the flow zone between inlet/outlet and the scaffold could shape the local flow near the scaffold outer surfaces. However, it had little effects on the flow distribution at the construct inner regions, largely due to the low permeability of the scaffold.

The study has also examined the flow condition when scaffolds had a smaller radius compared to the culture chamber. In such a case, flow channels were developed around the scaffold and flow tended to squeeze through the channels instead of passing through the scaffold evenly. To achieve homogeneous flow, such flow channels should be avoided and the size of the culture chamber should be matched with that of the scaffold.

The simulation has shown internal flow channels could lead to highly concentrated and elevated flow in the channel and neighboring construct regions. While cells could be highly sensitive to flow [131], cells near the flow channels could grow quite differently from that in other regions of the construct. Therefore, despite the improved flow in the construct regions near the channels, internal flow channels were not a complete solution to the mass delivery problem in 3D construct development. The flow channeling effects could be at least partially determined by the channel opening, orientation and size. Internal flow channels that had inlets facing incoming flow, orientation more aligned to the main flow stream, or larger sizes supported higher fluid flow. It is worth pointing out that all the internal channels in the study had one inlet on the construct left outer surface that faced the incoming perfusion flow. Such a setup was the prerequisite for the observed flow channel effects. In fact, when channels were oriented perpendicular to the perfusion flow and had both ends on the construct periphery surfaces, they hardly made any difference to the flow field in the construct (modeled results not shown).

While all the simulation in this work is based on the same inlet and outlet design, it can be expected that flow profiles in the bioreactor would be different if the inlet and outlet designs differ. Importantly, the flow profile would no longer be symmetrical about construct central radial plane, resulting in different flow stimulation to the two surfaces of the construct. This point can be well elaborated by Figure 3.13, which simulates the flow field when the bioreactor has the same setup as that in Figure 3.5d except that the outlet has a radius that is 2 mm bigger than that of the inlet.

Figure 3-13 Flow field in the bioreactor when outlet is 2 mm bigger than the inlet in radius

3.5 SUMMARY

Understanding the flow field induced by perfusion culture systems is fundamental for utilizing perfusion culture in 3D construct engineering. While the system designs could vary from experiment to experiment, this work has explored the flow

dependence on some common modular system designs. The obtained knowledge helps tissue engineers to evaluate the flow profile in perfusion systems and design perfusion systems for applying suitable flow stimulations to 3D constructs.

4 CHAPTER FOUR—A MATHEMATICAL MODEL FOR FLOW SHEAR-SENSITIVE 3D TISSUE CONSTRUCT DEVELOPMENT

4.1 INTRODUCTION

Dynamic culture not only improves the mass exchange between cells and the medium, but also exposes cells to physical stimulations [131-133]. It has proven to have important regulating effects on cell secretion, alignment, mobility and attachment [134-135]. For example, subjecting vascular smooth muscle cells and fibroblasts to dynamic culture has upregulated the cell expression of matrix metalloproteinase-1 (MMP-1), which in turn has promoted cell migration in collagen I gels [134]. To make full use of dynamic culture for tissue engineering, the cellular events in the process should be examined and the impact and interaction of different culture factors need to be characterized.

Existing CFD models on 3D construct development under dynamic culture have focused mainly on the biochemical effects especially of oxygen and glucose on the culture [136-139]. Some have also modeled the flow field and flow shear stresses around [140-141] or in the construct regions [108, 117, 142-143] to study the physical environment around cells. However, very few of these studies have correlated the flow induced physical stimulations, in particular, shear force with cell function (e.g., cell proliferation, attachment, and metabolism). Among the few studies, Lappa attributed organic tissue growth to shear force sensitive solute transport on the construct/liquid interface and proposed a parabolic dependence of the mass exchange flux at the construct surface on fluid shear [144]. While the simulation results agreed sufficiently well with experimental observations for tissue morphology evolution, the model did not involve the events in the internal regions

of the constructs [144]. In another study, Provin *et al.* modeled cell attachment on structured PDMS pillars of micro sizes in a mini perfusion bioreactor [145]. They suggested a linear relationship between local cell attachment ratio and shear stress when the shear stress was in the range of 0.2 Pa to 1 Pa. Outside the range, cell attachment was either unaffected (when <0.2 Pa) or totally prohibited (when >1 Pa) by the shear stress. By setting overall nutrient consumption of the biomass proportional to the retained cell number in the culture, this model indirectly related shear stress to cell metabolism. However, it did not engage the space-time evolution of the biomass [145]. More recently, Sacco *et al.* proposed a model that linearly linked flow shear stress with the maximum growth rate of cells in polymeric scaffolds under interstitial perfusion [146]. According to the simulation, the biomass production led to decreasing permeability thus resulting in higher local shear stress, which in turn promoted further biomass production. As a result, shear stress and biomass production were tightly coupled in a positive feedback loop, which could result in misleading results for the tissue growth at later stages of tissue development or high shear stress levels [146].

Having realized the importance of both biochemical and biophysical aspects of fluid flow to cell growth (Section 2.2) and the limitations of existing CFD models, this chapter develops a mathematical model taking into consideration both nutrient supply and shear stress for 3D tissue engineering. The developed model is expected to provide a more accurate simulation for the perfusion culture process compared to that only considered the nutrient effects. The work would help tissue engineers to better understand the cellular events in 3D constructs during dynamic culture at the cellular level.

4.2 MATHEMATICAL FORMULATION

4.2.1 MODEL SETUP

The simulation is conducted for an isotropic and homogeneous 3D construct under perfusion as shown in Figure 4-1. The construct is a porous rigid disc with radius L and thickness H . The radial and axial directions of the construct are denoted as x and y respectively. The perfusion medium is assumed to be Newtonian and incompressible. Temperature is taken as stable with negligible effects on medium properties during culture. Chondrocytes are used as the cell model.

Figure 4-1: Schematic drawing of the perfused construct

Flow enters the construct from the top surface and is symmetrical about the construct central axial axis. Thus the phenomena on the central axial plane of the construct are illustrative for the culture condition over the whole construct. To minimize computational cost, only half of the plane with four boundaries 1-4 as indicated in Figure 4-1 is modeled in the study.

The construct is regarded as a media comprising two phases (see the right side of Figure 4-1): matrix phase β and void phase α . The β phase comprises solid substances including scaffold material, cells adhering to the scaffold and deposited cell secretions. The void phase α is the fluidic region where the fluid flows. The

volume fraction of phase α in the construct is thus the porosity (ε) of the construct. During the culture process, the volume of β phase increases and porosity ε decreases along with cell proliferation and secretion. The matrix degradation within the modeled culture period is expected to be small and is thus neglected in the simulation.

4.2.2 VOLUME AVERAGING

With concerns on the biphasic structure of the construct, the volume averaging method is used in the model to derive spatially smoothed equations that govern transport phenomena in the porous media. l_α and l_β are characteristic dimensions for α and β phases in the averaging volume V (see Figure 4-1). To ensure the continuum of the averaged variables, the characteristic dimension of the representative elementary volume $2r$ must satisfy: $2r \gg l_\alpha$; $2r \gg l_\beta$; $2r \ll L$; $2r \ll H$. For tissue engineering constructs, $2r$ is expected to be around few hundred micrometers depending on the microstructure of the construct. In V , the β phase and α phase are assumed to be homogeneous. σ_α and σ_β are the respective volume fractions of α and β phase in V . They keep changing with time due to cell activities such as cell proliferation and protein deposition during construct culture, but always satisfy the condition:

$$\sigma_\alpha + \sigma_\beta = 1 \quad (4.1)$$

4.2.3 NUTRIENT TRANSFER

Fluid flow affects the distribution and mass exchange of various types of biomolecules in the culture. As dissolved oxygen (DO) is essential for cell metabolism needs but has very low solubility in culture medium compared to that in blood, DO is modeled as the limiting nutrient for cell growth in the study. The oxygen

concentrations in the β and α phases in the averaging volume V are denoted as C_β and C_α respectively.

In the β phase, DO concentration changes with cell metabolic activities and mass diffusion. $\sigma_{\beta 0}$ is the initial volume fraction of β phase. The volume fraction of β phase σ_β is the sum of the matrix material volume and cellular content volume. The model assumes 90% porosity and 10% matrix volume fraction before cell seeding with reference to that in Freed *et al.*'s experiment [147]. Thus the volume fraction of cellular content in V is represented by $\sigma_\beta - 0.1$ and the volume fraction of initial cellular contents in V is $\sigma_{\beta 0} - 0.1$.

Conservation of nutrient mass for β phase yields:

$$\frac{\partial C_\beta}{\partial t} = \nabla \cdot (D_\beta \cdot \nabla C_\beta) - R \cdot \frac{(\sigma_\beta - 0.1)}{(\sigma_{\beta 0} - 0.1)} \quad (4.2)$$

C_β is the DO concentration in β phase. The term on the left side is the change of mass concentration; the first term on the right side is the mass concentration change due to diffusion and the second term is the consumption of mass due to cell metabolism. D_β is the diffusion coefficient of DO in the β phase and is assumed constant during culture.

Michaelis-Menten equation relates the nutrient consumption rate with concentration of the nutrient [85]. It is used here to describe the cellular metabolic rate R :

$$R = \frac{R_m C_\beta}{K_m + C_\beta} \quad (4.3)$$

K_m is Michaelis constant; R_m is the maximum rate of oxygen consumption for seeded cells.

$$R_m = \rho_s^* R_m' \quad (4.4)$$

ρ_s is the cell seeding density and R_m' is the maximum oxygen uptake rate by single cells.

It is assumed there are no floating cells in the α phase. The mass exchange in the α phase thus only depends on mass diffusion and convection. Conservation of nutrient mass for α phase yields:

$$\frac{\partial C_\alpha}{\partial t} = \nabla \cdot (D_\alpha \nabla C_\alpha) - \nabla \cdot (U C_\alpha) \quad (4.5)$$

C_α is the DO concentration in the α phase. D_α is the diffusion coefficient of DO in α phase and assumed to be constant during culture. U is the local flow velocity. The second term on the right side is the mass change due to convection.

Equilibrium between the DO mass concentrations in β and α phases is assumed as:

$$C_\beta = K_{eq} C_\alpha \quad (4.6)$$

K_{eq} is the equilibrium coefficient.

Based on Equations (4.2) to (4.6), the conservation equation governing the DO distribution in averaging volume V is:

$$\frac{\partial}{\partial t} [(\sigma_\alpha + \sigma_\beta K_{eq}) C_\alpha] = \nabla \cdot (D_{eff} \nabla C_\alpha) - \frac{R_m K_{eq} C_\alpha}{K_m + K_{eq} C_\alpha} * \frac{(\sigma_\beta - 0.1)}{(\sigma_{\beta 0} - 0.1)} - \nabla \cdot (U C_\alpha) \quad (4.7)$$

D_{eff} is the overall effective diffusivity for V . Given that the transmembrane transport is instantaneous relative to the extracellular diffusive transport process, D_{eff} is set as described by Wood *et al.* [148]:

$$D_{eff} = D_\alpha \frac{2(1-\sigma_\beta)}{2+\sigma_\beta} \quad (4.8)$$

More sophisticated analytical formula for effective diffusivity can be found in the work of Ochoa *et al.* [149].

4.2.4 CELLULAR GROWTH

It is assumed that the cell proliferation and deposition are the two factors contributing to the expansion of the β phase. Both are assumed to follow the same growth kinetics. Mass conservation of cellular content in V yields:

$$\frac{\partial(\sigma_\beta - 0.1)}{\partial t} = K(\sigma_\beta - 0.1) \quad (4.9)$$

The term on the left side describes the overall cellular fraction change with respect to time. The term on the right side indicates the cell growth kinetics. $\sigma_\beta - 0.1$ is the volume fraction of cellular contents. K is the averaged specific growth rate. Modified Contois kinetics with consideration of both nutrient and cell contact inhibition effects has been found to describe cell kinetics more accurately as compared to the Moser and nth-order heterogeneous growth kinetic model [150]. Modified Contois kinetics is thus used in this study and is expressed as:

$$K = R_g \frac{C_\beta}{K_c \rho_{cell}(\sigma_\beta - 0.1) + C_\beta} - R_d \quad (4.10)$$

R_g is the maximum specific growth rate. K_c is the Contois saturation constant. R_d is the cell death rate. ρ_{cell} is the specific mass density of cell.

With reference to modified Contois kinetics, this work proposes a polynomial dependence of cell growth kinetics on flow shear stress based on previous reported experimental observations [151-152]. The final function for specific growth rate is modeled as:

$$K = R_g \frac{C_\beta}{K_c \rho_{cell} (\sigma_\beta - 0.1) + C_\beta} - R_d + (a\tau^{0.3} + b\tau) \quad (4.11)$$

Equation (4.11) effectively links cell growth with nutrient availability and flow shear stimulation. The third term on the right side in bracket describes the cell growth due to shear stress stimulation. τ is the local shear stress experienced by the cell. a and b are arbitrary constants. The determination of the a and b values is based on minimum discrepancy of simulation results with experimental observations. Based on the experimental data from Li *et al.* [151], it is derived that $a=1*10^{-6}$, $b=-1.5*10^{-6}$. With the proposed function, at low shear stress level, cell growth rate increases with shear stress and the increase rate decreases with shear stress. This indicates a diminishing impact of shear stress on cell growth as shear stress becomes higher. This is logical as cells are expected to gradually adapt to the shear stimulation and adjust their proliferation activity accordingly. Based on calculations, when shear stress exceeds around 0.1 Pa, the cell growth rate is slowed down reflecting impaired cell function under high shear stress. This critical value is in agreement with the literature that chondrocytes synthesized significantly higher amounts of collagen under 0.1Pa shear flow compared to static culture [152]. The calculations also show that when the shear exceeds 0.56 Pa, the cell number decreases, reflecting shear induced cell death. It is noted that the critical values of 0.1 Pa and 0.56 Pa depend on the values of constants a and b , which change with experiment setup.

For Newtonian fluids, shear stress τ is typically calculated as [153]:

$$\tau = \mu \cdot \left(\frac{\partial u}{\partial y} + \frac{\partial v}{\partial x} \right) \quad (4.12)$$

μ is the viscosity of the perfusion medium; u is the flow rate along x direction and v is the flow rate along y direction.

4.2.5 FLOW FIELD

In the biphasic construct, the medium flows through the interconnected pores in the α phase.

The medium density changes with nutrient consumption, metabolic waste accumulation and cell secretions during the culture period. However the change is usually very small and is negligible. This work thus assumes constant medium density. Conservation of flow mass yields:

$$\frac{\partial \sigma_\alpha}{\partial t} + \nabla \cdot U = 0 \quad (4.13)$$

Brinkman's equation [17] is used to describe the conservation of momentum in the porous construct:

$$\rho \cdot \frac{\partial (U * \varepsilon)}{\partial t} = -\nabla P - \mu \frac{(U * \varepsilon)}{K_d} + \mu \nabla^2 (U * \varepsilon) \quad (4.14)$$

ρ is the density of medium. K_d is the permeability of the construct. While U is the flow velocity in the α phase of the construct, $U * \varepsilon$ is the averaged flow velocity in the averaging volume. ε is the porosity of the construct and equals to σ_α or $1 - \sigma_\beta$. Carman-Kozeny equation is one of the most popular equations for calculating permeability of a random porous medium [154]. It is used in this work to describe the construct permeability K_d as [17]:

$$K_d = \frac{K_p \sigma_\alpha^3}{(1 - \sigma_\alpha)^2} \quad (4.15)$$

where K_p is a reference value.

4.2.6 BOUNDARY AND INITIAL CONDITIONS

As shown in Figure 4-1, the flow enters the construct region in the direction perpendicular to boundary 2. A parabolic flow is assumed on boundary 2 as: $U = -\frac{x}{L}\left(2 - \frac{x}{L}\right) * U_0$. U_0 is the flow rate along construct central axial axis in dimension of cm/s. Boundary 1 is the construct side surface in contact with the bioreactor. Non-slip boundary condition is assumed at the boundary 1 and the local flow velocity satisfies: $U = 0$. The central axial plane of the construct is symmetrical about boundary 3. Thus there is no local flow flux on boundary 3 and the flow at the boundary satisfies: $n * U = 0$. n is the unit vector normal to boundary 3. Boundary 4 is where the flow exits the construct thus flow pressure P on the boundary is equal to 0. The time required for flow to stabilize at the start of perfusion is not of concern in the model as it is relatively short to the entire culture process.

At boundaries 1, 3 and 4, there is no nutrient flux, thus $n * \nabla C_\alpha = 0$. At boundary 2, C_α is assumed to be constant and equal to the standard DO concentration in fresh medium c_0 , which is $0.199 * 10^{-6} \text{ mol/cm}^3$ [85, 120].

With seeding density ρ_s of $0.76 * 10^7 \text{ cells/cm}^3$ [147] and specific cell volume v_c of $549 \text{ } \mu\text{m}^3/\text{cell}$ [138], initial cellular fraction in the construct is calculated as:

$$\rho_s * v_c = 0.76 * 10^7 \text{ cells/cm}^3 * 549 \text{ } \mu\text{m}^3/\text{cell} = 0.00417$$

With 90% initial scaffold porosity, initial volume fraction of β phase $\sigma_{\beta 0}$ equals to:

$$\sigma_{\beta 0} = 0.00417 + (1 - 90\%) = 0.10417.$$

Cells are assumed to be seeded uniformly in the construct. Initial DO concentration in the α phase is set at $0.25 * c_0$. Initial velocity in the construct is 0.

4.2.7 NONDIMENSIONALIZATION

Nondimensionalization is performed to Equations (4.1) to (4.15) to reduce the number of parameters in the calculation and minimize computation costs.

Let $t = \frac{t'}{R_g}$, $x = H * x'$, $y = H * y'$, $C_\beta = c_0 * C'_\beta$, $U = U_0 * U'$, $u = U_0 * u'$, $v = U_0 * v'$, $P = \left(\mu \frac{U_0 H}{K_p}\right) * P'$. After nondimensionalization and reorganization, the overall

nutrient conservation equation derived from Equation (4.7) becomes:

$$(1 - 0.9\sigma_\beta)R_g \frac{\partial C'_\alpha}{\partial t'} = \frac{\nabla \cdot (D_{eff} \nabla C'_\alpha)}{H^2} + \left(0.9R_g \frac{\partial \sigma_\beta}{\partial t'} - \frac{R_m K_{eq}(\sigma_\beta - 0.1)}{(K_m + K_{eq} c_0 C_\alpha)(\sigma_\beta - 0.1)} - \frac{U_0}{H} * \nabla U'\right) C'_\alpha - \frac{U_0}{H} * U' \nabla C'_\alpha \quad (4.16)$$

The cell growth equation considering only the nutrient effect is

$$\frac{\partial \sigma_\beta}{\partial t'} = \left(\frac{C'_\alpha}{\frac{K_c \rho_{cell}(\sigma_\beta - 0.1)}{c_0 K_{eq}} + C'_\alpha} - \frac{R_d}{R_g} \right) (\sigma_\beta - 0.1) \quad (4.17)$$

The cell growth equation considering both the nutrient and the flow shear effects is

$$\frac{\partial \sigma_\beta}{\partial t'} = \left(\frac{C'_\alpha}{\frac{K_c \rho_{cell}(\sigma_\beta - 0.1)}{c_0 K_{eq}} + C'_\alpha} - \frac{R_d}{R_g} + a * \left(\frac{U_0}{H} \tau'\right)^{0.3} + b * \frac{U_0 \tau'}{H} \right) (\sigma_\beta - 0.1) \quad (4.18)$$

The nondimensionalized shear stress is

$$\tau' = \mu * \left(\frac{\partial u'}{\partial y'} + \frac{\partial v'}{\partial x'} \right) \quad (4.19)$$

The momentum equations are

$$-\frac{R_g H}{u_0} \frac{\partial \sigma_\alpha}{\partial t'} + \nabla \cdot U' = 0 \quad (4.20)$$

$$\frac{\rho R_g K_p}{\mu} \cdot \frac{\partial(U' * (1 - \sigma_\beta))}{\partial t'} + \frac{K_p}{K_d} (U' * (1 - \sigma_\beta)) = \nabla \cdot (-P'I + \frac{K_p}{H^2} \nabla(U' * (1 - \sigma_\beta))) \quad (4.21)$$

4.2.8 PARAMETER VALUES

The values of different parameters involved in the generated equations are summarized in Table 4-1. These values are selected based on the comparability of the system setup in the literature and the present study.

Table 4-1: Parameter values involved in the model

Parameters	Value	Units	References
Construct radius L	0.5	cm	[147]
Construct thickness H	0.307	cm	[147]
Standard DO concentration in fresh medium c_0	$0.199 * 10^{-6}$	mol/ cm ³	[85, 120]
Equivalent coefficient K_{eq}	0.1		[150]
Diffusion coefficient of oxygen in β phase D_β	$8.6 * 10^{-7}$	cm ² /s	[155]
Diffusion coefficient of oxygen in α phase D_α	$3 * 10^{-5}$	cm ² /s	[155]
Maximum oxygen uptake rate by single cells R_m'	$1.86 * 10^{-18}$	mol/(cell s)	[156]
initial cell density ρ_s (with 45% seeding efficiency)	$0.76 * 10^7$	cells/cm ³	[147]
Maximum rate of oxygen consumption for seeded cells $R_m = R_m' * \rho_s$	$1.41 * 10^{-11}$	mol/(cm ³ s)	
Michaelis constant K_m	$5 * 10^{-9}$	mol/cm ³	[155]
Maximum specific growth rate R_g	$8 * 10^{-6}$	s ⁻¹	Curve fitting for [147]
The specific cell mass density ρ_{cell}	0.182	g/cm ³	[17]
The modified Contois saturation constant K_c	$1.18 * 10^{-5}$		Curve fitting for [147]
The cell death rate coefficient R_d	$9 * 10^{-7}$	s ⁻¹	Curve fitting for [147]
Density of medium ρ	1.009	g/cm ³	[85]
Dynamic viscosity of the medium μ	$8.4 * 10^{-3}$	g/(cm-s)	[85]
Reference value for permeability of the porous media K_p	10^{-11}	cm ²	[24]
Specific cell volume v_c	549	μm ³ /cell	[138]

4.2.9 STUDY DESIGN AND CALCULATION

According to the model, the cell mass under perfusion can be directly affected by flow rates and is shear-sensitive. Two comparative studies are carried out with the proposed model:

1. Construct development under different perfusion rates. Simulation results with two perfusion flow rates, 0.002 cm/s and 0.02 cm/s, are compared to

study the flow rate effect on cell growth. The two flow rates are chosen with reference to the *in vivo* interstitial flow magnitude which is around 4 $\mu\text{m/s}$ [104]. Static culture condition is also simulated for comparison.

2. Construct development under the same perfusion rate. Simulation results with and without concerns of shear stress are compared to study the significance of considering shear stress in dynamic culture analysis.

The culture time is set as 25 days. The Equations (4.16) to (4.21) are solved using Femlab 3.4 (Comsol). In total 8678 elements with 188 boundary elements are used for meshing the construct. The minimum element quality is 0.5747. The calculation convergence is based on 1% discrepancy of the calculated results.

4.3 RESULTS AND DISCUSSION

4.3.1 CONSTRUCT DEVELOPMENT UNDER DIFFERENT PERFUSION FLOW RATES

In static culture, fresh medium diffuses into the inner construct region from the scaffold top surface. Figure 4-2 shows the matrix phase (upper side) and DO (lower side) distribution in the construct after 25 days of static culture. The x and y axes of the figure are the boundary 4 and boundary 1 of the modeled plane respectively as indicated in Figure 4.1, Section 4.2.1. The colors in the figure indicate the value of matrix volume or DO concentration. It is noted that the coordinates and matrix volume/DO concentration in the figure are all dimensionless due to the nondimensionalization process as discussed in Section 4.2.7. Distinctively, more cells grow near the upper boundary region (upper side), which is in correspondence with the DO distribution profile (lower side). Both cell number and DO concentration level decrease with depth in the scaffold. The uneven cell distribution

indicates that in static culture, the nutrient transport into the inner scaffold region through only diffusion is inadequate to meet the needs for local cell growth.

Figure 4-2: (Upper) matrix phase and (lower) dissolved oxygen level in the construct under static culture on day 25

Under dynamic culture, fresh medium is fed from the construct's top surface and forced through the construct. It exits from the construct's bottom surface. As a result, cells are exposed to enhanced nutrient transport and interstitial flow stress.

Figure 4-3 presents the simulated matrix phase (upper side) and DO (lower side) distribution in the construct at the end of culture under flow rate $U_0=0.002$ cm/s.

Figure 4-3: (Upper) matrix phase and (lower) dissolved oxygen distribution in the construct on day 25 with $U_0=0.002$ cm/s

Compared to static culture outcome in Figure 4-2, much more cells and higher nutrient levels are observed in Figure 4-3, showing the important contribution of dynamic culture in improving construct development. The cell and nutrient distributions also appear more even. Calculations show the average and maximum

flow shear stresses in the construct on day 25 reach 1.28×10^{-4} dyn/cm² and 0.009 dyn/cm² respectively.

Figure 4-4 shows the matrix phase (upper side) and DO (lower side) distribution in the construct at the end of perfusion culture when $U_0=0.02$ cm/s. Compared to the conditions of static culture (Figure 4-2) and lower perfusion rate (Figure 4-3), there are more cells and DO level is higher across the construct. The distributions of cells and oxygen are also more even. Compared to that at flow rate $U_0=0.002$ cm/s, shear stresses at $U_0=0.02$ cm/s are slightly higher in the construct with an average value of 2.27×10^{-3} dyn/cm² and maximum value of 0.089 dyn/cm² on day 25 culture. on day 25 culture. Thus, the faster expansion of the cell mass in the construct at $U_0=0.02$ cm/s can be related to the improved nutrient supply and the increased shear stimulation induced by the higher perfusion rate.

In constructs under both perfusion flow rates, the matrix phase has relatively larger volume fractions at the construct top and periphery regions. This profile can be related to the flow shear stress distribution, which also has higher values at the scaffold top and periphery regions (data not shown) based on the calculation. Thus, under the given setup, both nutrient supply and shear stress have affected the cell growth and distribution in the construct.

Figure 4-4: (Upper) matrix phase and (lower) dissolved oxygen distribution in the construct on day 25 with $U_0=0.02$ cm/s

Calculation shows that the values of average shear stress in constructs under both flow rates increase very slowly with time during the culture process. This can be explained as: with increasing cell mass, the permeability of the construct K_d decreases (based on Equation (4.15)), leading to a gradually increasing flow shear stress. However, despite the cell mass increase, the scale of cellular volume fraction in the construct is still relatively small, limiting its influences to the flow field.

To compare the culture outcome under different perfusion flow rates quantitatively, the averaged volume fraction of cellular mass $\overline{\sigma_\beta}$ and DO concentration in the construct $\overline{C'_\alpha}$ are determined as follows:

$$\overline{\sigma_\beta} = \frac{\int_{x'=0}^{1.63} \int_{y'=0}^1 \sigma_\beta dx' dy'}{\int_{x'=0}^{1.63} \int_{y'=0}^1 dx' dy'} - 0.1 \quad (4.22)$$

$$\overline{C'_\alpha} = \frac{\int_{x'=0}^{1.63} \int_{y'=0}^1 C'_\alpha dx' dy'}{\int_{x'=0}^{1.63} \int_{y'=0}^1 dx' dy'} \quad (4.23)$$

The calculated results for day 25 culture are compared in Figure 4.5. The volume fraction of cellular mass in the construct under static culture reaches 0.03454 on day 25 from an initial value of 0.00417. The values are much higher under perfusion culture: 0.07408 for $U_0=0.002$ cm/s and 0.08807 for $U_0=0.02$ cm/s. The averaged DO level in the construct is 0.54754, 0.88197 and 0.98540 with static culture, $U_0=0$, 0.002 and 0.02 cm/s respectively. The comparison demonstrates that the cell proliferation and nutrient supply can be significantly improved with flow rate even as low as 0.002 cm/s. Furthermore, the higher the flow rate, the faster cells proliferate and the higher the DO concentration level in the culture.

Figure 4-5: Comparison of averaged (left) volume fraction of cellular mass and (right) dissolved oxygen concentration in the construct after 25 days of culture under different perfusion rates

When flow increases from 0 to 0.002 cm/s, there is significant elevation in both nutrient supply and flow shear. In comparison, when flow rate increases from 0.002 cm/s to 0.02 cm/s, there is only very small increase of nutrient supply in the construct while the shear stress has a 10 fold increase. It is observed that the impact on cell number by increasing flow rates from 0 to 0.002 cm/s is much more significant than that by elevating flow rates from 0.002 cm/s to 0.02 cm/s despite that the flow change at the former case is smaller. The observation is thus likely due to the greater nutrient supply change in the former case. While the much higher shear stress increase in the latter case is supposed to promote cell growth, its influence appears to be relatively smaller compared to that of nutrient supply increase caused in the former case under the given setup.

4.3.2 FLOW SHEAR EFFECTS FOR CELL GROWTH

Figure 4-6 presents the simulated cellular matrix phases in the construct after 25 days of 0.002 cm/s perfusion with (upper side) and without (lower side) considerations of flow shear effects. Figure 4-7 presents the corresponding DO concentration distribution in the construct.

Figure 4-6: Cellular matrix phase in construct at day 25 culture with $U_0=0.002$ cm/s, with (upper) and without (lower) considerations of flow shear effects

Figure 4-7: Dissolved oxygen in construct at day 25 culture with $U_0=0.002$ cm/s, with (upper) and without (lower) considerations of flow shear effects

Based on Figures 4-6 and 4-7, the consideration of shear stress brings in very limited difference to the distributions of cells and DO in the construct compared to that without shear consideration. However, based on the calculated results as shown in Table 4-2, with shear consideration, the volume fraction of the cellular mass is higher and the averaged DO concentration is lower in the construct than that without shear consideration. The higher cell number growth is in agreement with

the lower DO concentration as more cells would consume more oxygen. The culture outcomes under flow $U_0=0.02$ cm/s with and without shear consideration are also given in Table 4-2 and similar comparison results are obtained.

Table 4-2: Averaged cellular mass volume fractions and dissolved oxygen concentrations at day 25 dynamic culture with and without considerations of flow shear effect

	$U_0=0.002$ cm/s		$U_0=0.02$ cm/s	
	w/o shear	w shear	w/o shear	w shear
Averaged cellular mass volume fraction	0.07039	0.07408	0.07958	0.08807
Averaged dissolved oxygen concentration	0.88792	0.88197	0.98691	0.98540

It is noted that the difference to nutrient concentration level in the construct due to flow shear consideration is minor for both perfusion rates. This could be because the nutrient level in the concerned dynamic culture is very close to that in fresh medium even when shear stress is considered. In comparison, shear stress consideration brings in a much bigger difference to cell mass, indicating the important roles of shear stress in promoting cell proliferation when nutrient supply is relatively constant.

The comparison between the cultures under the two different flow rates shows that shear effect on promoting cell growth is more significant at higher flow rates. This could be because at higher flow rates, the flow induced shear stress is higher.

4.3.3 COMPARING MODELING RESULTS WITH REPORTED EXPERIMENTAL DATA

The proposed model examines the stimulating effects of both fluid shear and nutrient level for cell growth kinetics. The modeling results are compared with Li *et al.*'s experimental work which investigated the flow shear effect on bone construct development [151]. Li *et al.*'s work showed that when mass transport was kept constant, tissue volume and nutrient consumption rate increased consistently with

shear stress, exhibiting the direct shear impact on improving cell growth and nutrient consumption. The work also showed that, at the same nutrient level, higher shear stress supported higher cell number [151]. Thus, the proposed shear-sensitive cell growth model of this work is in agreement with the experimental observations [151].

Li *et al.*'s work also observed that when the flow shear stress was kept constant, mass transport brought very little changes to the glucose consumption of the culture [151]. This indicated that the cell metabolism rate of individual cells was not affected by the change of nutrient supply in the given setup. Thus it can be extrapolated that the increased nutrient consumption observed with increased shear stress was likely due to the improved cell proliferation and higher cell number, rather than the metabolism rate of individual cells. The proposed model in this work also matches the extrapolation.

4.3.4 LIMITATIONS OF THE MODEL

This work proposes a shear dependent function for cell growth with coefficients arbitrarily determined based on published literature data. It is expected that the coefficient values differ with culture conditions such as cell types and medium properties. These values should be appropriately selected for individual experiments.

The Brinkman equation used in the proposed model is an averaged calculation for describing flow field. It simulates the averaged shear stresses at the local regions. This saves computational cost but leads to information loss at a micro-level. To improve the accuracy of the model, the Navier-Stokes equation can be utilized.

The proposed model assumes even cell distribution after seeding. In actual seeding practice for 3D constructs, such cell distribution is however very difficult to obtain. Instead, cells usually aggregate on the outer layers of the constructs and it is difficult for them to migrate into the inner regions due to the blockage by construct material at the outer regions of the scaffold. Uneven cell distribution in constructs is thus more common in practice and should be taken into account in further model development.

It is worth pointing out that though the structure of the construct at the micro-level could vary from site to site, this work assumes homogeneous porosity and permeability of the construct. The assumption is based on that scaffolds prepared for cell seeding are preferred to possess even structure, which can be reasonably achieved as shown in Figure 6.6, Section 6.4.2.1. Moreover, as Brinkman model is applicable to porous medium with a mix of pore configurations, the possible minor variance between the pore configurations is well tolerated by the model [157]. In cases where the porosity and pore configuration are purposely designed to differ greatly across the construct [158], the model setup at Section 4.2.1 of this work can be varied accordingly and Brinkman model can still be applied for individual porous regions of similar pore configurations. The specification and control for porous architecture of the construct could be obtained by advanced scaffold fabrication methods such as additive manufacturing [129].

4.4 CONCLUSION

Dynamic culture improves cell growth through nutrient supply enhancement and physical stimulation to cells. This work has successfully developed a computational model that simulates the effects of both flow induced nutrient supply and shear

stress for dynamic culture supported 3D construct engineering. The modeling results revealed that dynamic flow, even at very low flow rate, can support much better mass exchange, higher cell number and more even cell distribution compared to static culture. Higher fluid flow promoted further nutrient supply and mass exchange in the culture, resulting in a better growth environment and improved cell proliferation compared to lower flow rate. While nutrient supply dominated shear stress in promoting cell proliferation at low shear stress levels, shear stress had increasing importance in promoting cell proliferation when flow rate was increased. Calculations with shear effect consideration predicted higher cell number growth and lower DO concentration in the construct compared to that without shear consideration. The proposed models of this work assist tissue engineers to better understand cell-flow relationship in perfusion culture at the micro-level.

5 CHAPTER FIVE—PERFUSION BIOREACTOR SYSTEM DEVELOPMENT

Perfusion bioreactors provide a convenient tool for developing 3D bioconstructs and studying cell behavior under dynamic flow *in vitro*. This chapter presents the development of a novel perfusion bioreactor for 3D tissue engineering.

5.1 DEVELOPMENT OF A NOVEL PERFUSION BIOREACTOR

In this work, a novel perfusion bioreactor is designed and constructed for fibroblast based 3D construct development. The ultimate goal of the bioreactor design is to reliably and reproducibly deliver a dynamic culture environment which enables functional fibroblast growth and 3D soft tissue development.

5.1.1 DESIGN CRITERIA

Several essential criteria are set for the design of the perfusion bioreactor.

First, it should enable nutrients (e.g., glucose, amino acids) to be delivered to and metabolic waste to be removed away from cells on time so as to provide a favorable and consistent cell growth environment. As passive diffusion is ineffective for solute exchange between cells and its milieu especially in the inner region of the scaffold, continuous perfusion through the scaffold is needed to facilitate local solute diffusion and exchange.

Secondly, an unstable culture environment could result in abnormal cell functions. Therefore, the perfusion bioreactor should ensure a stable and suitable culture environment for cells, e.g., with respect to the solute concentration in the medium, dissolved oxygen concentration, temperature and other stimulations such as

physical stimulation. In addition, because cells likely need time to adapt to the culture environment similar to the maturation of tissues (e.g., the maturation of a tissue may take up to 3-4 months [159]), the bioreactor should allow for long term and consistent cell culture.

Thirdly, flow rate, frequency and culture duration have important impacts on the IF profile, corresponding to specific cell responses in the fluidic growth environment. The system should be highly flexible and allow easy adjustment to these flow parameters during the culture process. *In vivo*, the interstitial flow is relatively small (between 0.1 and 4 $\mu\text{m/s}$ in humans [26]) and its frequency change is dependent on the body movement (e.g., resting, running, eating). The design of the bioreactor should benchmark the *in vivo* situations and provide IF profiles approximating those *in vivo*.

Fourthly, while the culture environment is highly dependent on the bioreactor design and setup, it can also be changed by the evolving scaffold architecture and cell activities. It is important to monitor the culture easily and timely so as to ensure the culture is on the right track. Thus the bioreactor system should allow easy visual monitoring during the culture process.

Fifthly, in order to avoid abnormal cell growth and culture, the system should support easy sterilization and contamination-free culture.

Sixthly, to simplify handling process, the system should allow simple and easy loading and harvest of constructs.

Finally, the culture chambers of the bioreactor system where constructs are located should be compact enough to be fitted into a standard commercial culture incubator. This is to ensure suitable and consistent temperature, moisture and CO_2 levels for

the culture. It can also help reduce the bioreactor development cost by utilizing common laboratory facilities.

5.1.2 FLOW CIRCUIT DESIGN

Figure 5-1 presents the schematic of the flow circuit in the developed perfusion system. Four main parts, the pulsatile pump, the flow meter, the bioreactor chamber and the medium reservoir, are connected via oxygen permeable tubings.

Figure 5-1: Flow circuit of the developed perfusion bioreactor system

1) Pulsatile pump

A peristaltic pump (Heidolph SP quick, PD5201) is used to deliver pulsatile flow to the cultured construct. The flow rate and frequency can be set automatically. The lowest flow rate can go to below 1 ml/min. The flow frequency can be flexibly adjusted with the hose size. Temporal flow rate is displayed on the liquid crystal display (LCD) of the pump.

2) Flow meter

The flow meter (Cole-Parmer, KH-03217-6Z) serves two main purposes: firstly to check if the pump delivers an output as shown on its LCD display and secondly as an online monitor for the flow change in the culture. The flow change is an indirect parameter of the tissue construct permeability, which is an important reflection of tissue remodeling.

3) Bioreactor chamber

The chamber is where cultured constructs are placed. It is designed to fit within a standard commercial incubator (5.0% CO₂, 36.9°C) during culture process. Its design and fabrication are described in detail in Section 5.1.3.

4) Medium reservoir

The perfusion system is to be operated continuously as a closed system. The flow starts from and returns back to the medium reservoir after one round of circulation through the pump, flow meter and bioreactor chamber. In order to maintain relatively stable solute concentrations (e.g., glucose, amino acids, metabolic waste), the medium will be provided in a sufficiently large quantity to buffer the effect of any concentration changes due to cell activities. The medium can also be changed in batches for long-term culture. The medium can be sampled during culture to check the medium condition and ensure cells are fed with medium of proper standards.

5.1.3 CULTURE CHAMBER DESIGN

The design of the culture chamber is shown in Figure 5-2. It contains four main parts: the top chamber, the scaffold retainer, the retainer cover and the bottom chamber. Medium flows into the bioreactor from the inlet of the top chamber, passes through scaffold restrained in the scaffold retainer and exits the bioreactor from the outlet at the bottom chamber.

locking slip and the bottom of the retainer are highly porous in order to eliminate their interference to flow stream.

The assembly of the two chamber parts is based on a simple press fit mechanism, which requires little handling effort. To ensure a leak-proof assembly, sealing tape can be applied at the assembly interface as needed. A flow zone is designed between the chamber inlet/outlet and the positioned scaffold inside the chamber in order to obtain homogeneous flow profile over the cross section of the scaffold based on the simulation study described in Chapter 3.

All the chamber components are machined from polycarbonate, which is chemically inert and transparent. The material ensures biocompatibility of the chamber and facilitates direct visual inspection to the culture in the chamber. It also enables easy sterilization of the chamber by autoclaving.

To supply oxygen to cells seeded inside the scaffold, highly oxygen-permeable silicone tubing (MasterFlex, Cole-Parmer) is used to connect the flow circuit.

The detailed dimensions of the scaffold retainer, retainer cover, top chamber and bottom chamber are shown in Figures 5-3 to 5-6.

Figure 5-3: Dimensions of scaffold retainer

Figure 5-4: Dimensions of retainer cover

Figure 5-5: Dimensions of top chamber

Figure 5-6: Dimensions of bottom chamber

The height of the scaffold container is 5 mm, which corresponds to the thickness of the thickest 3D construct reported in literature [121]. For scaffolds that are less than 5 mm thick, the size of the scaffold container could be modified accordingly during bioreactor design. Thus, there would be no concern about scaffold tilting and the induced flow profile change for this work.

Based on the findings in Section 3.3.2, bigger buffer zone results more even flow distribution over the construct surface. Thus bioreactor is purposely designed with a buffer zone of 20 mm long. This is expected to achieve homogeneous flow profile in the construct region and the flow simulation in this fabricated bioreactor is given in Section 5.2. It is noted that the selection of length of the flow buffer zone would not affect the outcome of simulation results in Section 3.3.2.

The radii of scaffold and chambers are determined based on the size of wells of standard 24 well culture plates, which are used for the fabrication of scaffolds in this project. The size of the flow inlet/outlet is based on the size of commercially available polystyrene tubing adapters, which are used to connect the chamber with the tubing in the flow circuit.

5.1.4 CHAMBER FABRICATION AND ASSEMBLY

The machined components of the culture chamber and their assembly are shown in Figure 5-7: (a) shows the top chamber; (b) is the bottom chamber; (c) shows the scaffold retainer and the retainer cover; both retainer and retainer cover are designed with thorough holes to minimize flow interruptions; (d) shows the scaffold retainer press fitted in the bottom chamber; (e) shows the overall assembly of the culture chamber; (f) displays the culture chamber connected with tubings through commercial tubing adapters.

Figure 5-7: Machined chamber components and their assembly

5.1.5 PERFUSION SYSTEM ASSEMBLY

The final assembled perfusion system is as shown in Figure 5-8.

Figure 5-8: Assembly of perfusion culture system—one culture chamber

During actual culture process, in order to minimize the risk of contamination, a sealed centrifuge tube as shown in Figure 5.9 could be utilized as the culture medium reservoir.

Figure 5-9: Medium reservoir used in actual culture practice

The culture capacity of the perfusion system can be scaled up by adding more chambers in parallel or sequence in the flow circuit as schematically shown in Figure 5-10.

(a) Perfusion system with multiple culture chambers in parallel

(b) Perfusion system with multiple culture chambers in sequence

Figure 5-10: Assembly of perfusion culture system with multiple culture chambers in parallel (a) and sequence (b)

5.2 MECHANICS OF FLOW IN THE PERFUSION BIOREACTOR

The flow profile in dynamic culture has direct impact on the growth and function of cultured cells and tissues, such as cell proliferation, ECM component secretion [160], morphology [161], cellularity [54], tissue strength and biocompatibility [162]. The flow mechanics supported by the constructed perfusion bioreactor is simulated in this section.

5.2.1 FLOW SPACE AND SETUP

Figure 5-11 shows the culture space in cylindrical bioreactor chamber. In (a), AA is the axial symmetrical plane of the culture space. (b) shows plane AA and indicates its symmetrical axial axis. (c) presents the culture space on the symmetrical plane AA. The flow is supplied from inlet down to the chamber. After an expansion in the upper stream flow zone, it reaches the scaffold. It then passes the scaffold, propagates through the downstream flow region and converges to the outlet.

Figure 5-11: Assembled bioreactor and flow space on its symmetrical plane.(a)

Assembled bioreactor; (b) symmetrical plane of the bioreactor; (c) culture space on the symmetrical plane. Arrows in (c) indicate flow directions.

The perfusion parameters used in the culture are given in Table 5-1. The permeability and porosity value of collagen matrix are adopted from literature for the present system due to the similar fibrous structure of the scaffolds [163-164]. Temperature of modeling is set at 37°C, which is the common culture temperature for mammalian cells and also the body temperature of humans. The properties of

standard culture medium are available and adopted from literature [165]. The input flow rate 1 ml/min is an arbitrary value chosen in the culture experiment, which equals to an average 0.083 mm/s in the construct region. This flow rate is higher compared to that of the work by Ng *et al.* [52], in which flow-induced cell response was already obvious. Thus the rate set in the model can be expected to induce observable influence on cell growth in the developed bioreactor.

Table 5-1: Parameters involved in fluid mechanics

Parameters		value
Temperature		37°C
Flow space	Diameter of inlet and outlet	0.41 cm
	Diameter of upper stream flow path D1	1.3 cm
	Diameter of downstream flow path D2	1.3 cm
	Length of upper stream flow region L1	2 cm
	Length of downstream flow region L2	2 cm
Scaffold	Diameter of scaffold Ds	1.3 cm
	Length of scaffold region Ls	0.5 cm
	Permeability	$4 \times 10^{-7} \text{ cm}^2$ [164]
	Porosity	90% [163]
Medium	Density of fluid ρ	1 g/cm ³ [166]
	Dynamic viscosity of the fluid μ	$8.4 \times 10^{-3} \text{ g/(cm-s)}$ [85]
Flow	Volume rate in the chamber inlet Q	1 ml/min or 0.016 ml/s

5.2.2 METHODOLOGY

The fluid flow and pressure fields inside the chamber are simulated with the commercial software Femlab 3.1 (Comsol). Boundary conditions and solver are specified according to the system setup and design. It is assumed that the fluid is Newtonian and continuous with non-slip boundary conditions, which are commonly applied for modeling work in tissue engineering [138-139, 167].

5.2.2.1 Governing equations of simulation

In the porous scaffold region, Brinkman's equation is used to calculate the pressure and velocity of the flow [118, 168]. In the upper stream and down stream flow regions (see Figure 5-11c) of the culture space, free flow is present and Navier-Stokes equation is used.

It is noted that the matrix permeability changes along with the dynamic cell growth and tissue remodeling process. When cell concentration is low, the permeability of the arterial tissue is determined by the permeability of the fiber matrix. However, when the volume fraction of cells increases, the influence from cells to matrix permeability becomes increasingly prominent [169]. The modeling work in this section assumes consistent matrix structure, which represents the conditions at the starting stage of the culture.

5.2.2.2 Software utilization

The geometry of the culture chamber with scaffold located inside is created and meshed by Femlab 3.1 (Comsol). As the flow within the chamber is symmetrical about the central axial axis, only the axial symmetry plane of the culture space is studied. The meshed plane is shown in Figure 5-12. A total number of 6632 elements are used for the mesh and the minimum element quality is 0.5930. Above this meshing density, the calculation results are independent of the meshing density with a discrepancy smaller than 0.00014. Thus, it is confident that this meshing density satisfies the mesh convergence criteria.

Figure 5-12: Meshed symmetry plane of the culture space

In order to equalize the viscous momentum transport between the porous and the free flow region, viscous stress tensor is used for both Navier-Stokes and Brinkman's equation during calculation.

The flow profile at the inlet is set as parabolic with flow rate $U(r)$ equal to $U_{max} * (1 - r_{in}^2 / R_{in}^2)$ as depicted in Figure 5-13, with U_{max} being the flow rate along the central axial axis of the inlet, r_{in} being the distance from the central axial axis and R_{in} being the inlet radius.

Figure 5-13: Flow velocity at the flow inlet

5.2.3 SIMULATION RESULTS

5.2.3.1 Velocity field

The simulated velocity profile on the symmetry plane of the culture space is shown in Figure 5-14.

Figure 5-14: Flow velocity profile in the perfusion chamber

Based on the simulation, while perfusion velocity varies at different regions of the culture space, the overall velocity in the chamber remains at a fairly low value, due to the small flow input. The highest perfusion rates concentrate at the inlet and outlet corresponding to the smallest cross sectional areas at these two locations. Flow from the inlet faces a sudden expansion of flow path upon entering the chamber. In the upper stream flow zone between the inlet and the scaffold, the flow rate gradually slows down and becomes more even along its way to the scaffold. Upon reaching the scaffold surface, flow distributes fairly evenly over the scaffold cross section.

Figure 5-15 plots the flow profile on the horizontal plane of the scaffold which is 0.3 mm away from the scaffold top surface.

Figure 5-15: Plot of flow velocity on the scaffold plane that is 0.3 mm from the scaffold top surface

As shown in Figure 5-15, the flow across the plane is rather homogeneous except the scaffold-chamber interface where some flow disturbance is present. The flow is around 5.6×10^{-4} cm/s. Simulations have also been carried out and shown similar homogenous flow profiles on planes that are 0, 0.1, 0.4, 0.5 mm away from the scaffold top surface (results not shown). The even flow distribution in the scaffold is expected to facilitate even construct development.

5.2.3.2 Pressure

The simulated fluid pressure in symmetrical plane of the culture is presented in Figure 5-16.

Figure 5-16: Flow pressure in the culture space

The pressure profile in the culture space is characterized with three distinctive regions: a consistent and relatively high value at the upper flow region, a transition zone in the scaffold region and a consistent and relatively low value in the downstream flow region. In the transition zone, flow pressure drops gradually, attributed to the flow resistance of the porous matrix.

Figure 5-17 presents the pressure profile along the central axial axis of the scaffold. It shows the pressure reduces steadily from the scaffold top surface to bottom surface. While the homogeneous flow velocity in the scaffold region indicates consistent dynamic pressure across the scaffold, the observed pressure drop in Figure 5-17 is mainly due to the change of static pressure. Simulation work has also indicated similar pressure profiles along other axial axis of the scaffold (0.2, 0.4 and

0.6 mm from the central axial axis) (data not shown), conforming to the even flow profile on the same radial plane of the scaffold as observed in Figure 5-15.

Figure 5-17: Pressure across the scaffold along central axial axis

It is noted that the simulation for flow mechanics in the bioreactor does not consider the possible blocking effects of scaffold retainer. Figure 5.18 presents the flow distribution in the construct region when the presence of scaffold retainer is considered. It indicates that flow distribution across the construct is affected by the retainer especially at the corners of the interface between the scaffold and the retainer. However, as shown in Figure 5.19, a closer look at the flow velocity on different radial planes of the construct demonstrates that the flow variances between different radial planes are limited to the construct periphery regions and within 0.1×10^{-3} cm/s. While in most parts of the construct, flow is relatively consistent around 0.9×10^{-3} cm/s, the variance of flow field at the interface regions between the construct and scaffold retainer is around 10%. Thus the effect of

presence of scaffold retainer to the flow field is considered small and neglected in the work.

Figure 5-18: Flow field in the construct region when scaffold retainer is present

Figure 5-19: Flow field on different radial planes of the construct (0.05, 0.15, 0.25, 0.35, 0.45 mm from the construct top surface) when scaffold retainer is considered. Red line represents the flow field on the scaffold plane that is 0.05 mm away from the construct top surface.

5.3 DISCUSSION

A pulsatile perfusion bioreactor system that allows flexible adjustment of flow rate, frequency and duration, easy scaffold loading and harvest and online culture monitoring has been designed and constructed for 3D biconstructs development. The flexible adjustment of flow rate, frequency and duration of the system assures the easy manipulation of perfusion stimulations. Easy system assembly and construct loading and harvest reduce the handling requirements in the culture process. The transparency of chamber materials supports real-time observations of the cultured constructs, making it convenient to study the tissue culture status during culture process. The inert and autoclavable properties of the construction materials meet the needs for easy sterilization and contamination-free culture.

The developed system has fulfilled several goals:

- 1) To allow easy adjustment of flow rate, frequency and duration for cell culture

This is done via a pulsatile pump, which allows automatic adjustments of flow rate and frequency in a range approaching that of *in vivo*. Long term of continuous culture is also enabled to allow studying cell adaptation under flow stimulation.

- 2) To allow easy construct loading and harvest

This is done by making the chamber an assembly from two separated pieces, the “top chamber” and “bottom chamber”. Ease of assembly and disassembly of the two pieces is achieved with a simple press fit mechanism. The scaffold is kept at the interface region of the two chamber components so that it can be easily put in and taken out of the chamber.

- 3) To keep scaffold in the fixed position in the culture chamber under perfusion

Medium enters the chamber through the inlet in top chamber and flows out through the outlet in bottom chamber. To prevent scaffold from floating away under the flow pressure, a scaffold retainer is designed to hold the scaffold at the assembly interface of the culture chamber.

- 4) To expose scaffold cross sections to the same level of flow stimulation

This is achieved by designing a flow zone between flow inlet/outlet and the scaffold inside the culture chamber. The flow agitated by sudden flow path expansion at the chamber inlet can be stabilized in the buffer zone before reaching the scaffold.

- 5) To enforce medium flow through scaffold and avoid hypoperfusion

This is done by matching the radius of the scaffold with that of the culture chamber.

- 6) To allow easy monitoring of the culture and construct condition

The culture conditions such as pH and glucose concentration can be monitored by taking samples from medium reservoir. Visual observations are achieved by utilizing transparent and inert polycarbonate material for constructing the chamber walls.

- 7) To maintain culture temperature and pH of the culture medium at the physiological level

This is achieved by locating the culture chamber inside a commercial cell culture chamber (5.0% CO₂, 36.9°C) during perfusion and selecting culture reagents with suitable concentrations of bicarbonate.

- 8) To allow easy connections with tubing in the flow circuit

This is done by the flow adapter design at the inlet of the top chamber and outlet of the bottom chamber. Commercial flow adapters are used to connect the chamber to the other circuit components.

9) To allow easy sterilization and long term contamination-free culture

To ensure sterility of the system, all the construction materials are autoclavable. The whole chamber is also designed to be compact enough to be located inside a commercial cell culture incubator to lower the risk of contamination during culture.

The fluid mechanics in the perfusion chamber has been modeled. Commercial CFD software has been used to solve the governing equations and generate visual presentations. Overall, with the low volume flow rate set for the study model, the flow is laminar with low levels of flow rate and pressure in the culture space. The scaffold has a distinct obstruction effect to the flow, as reflected in the sharp pressure drop along the flow propagation path. With the dampening effects of the upper flow region, flow reaching scaffold spreads evenly over the scaffold surface. The flow velocity does not differ significantly over the entire scaffold region and the flow pressure differences in the scaffold are largely due to the static pressure difference. The flow stimulation confirms that the developed perfusion bioreactor system provides even flow stimulation to 3D constructs.

It is worth mentioning that despite the advantages of conical inlet in delivering even flow profiles across the scaffold surface as simulated in Section 3.3.1, the bioreactor used the cylindrical inlet design in this work. This is because cylindrical inlet when combined with the large flow buffer zone is capable of helping deliver a homogeneous flow. In addition, cylindrical inlet is easier to be machined compared to the conical design and there is no compromise to the culture process when the

cylindrical design is utilized. Thus, on the basis that the ultimate criteria of obtaining homogeneous flow stimulation for scaffold growth can be fulfilled, the cylindrical inlet is chosen in this work.

5.4 SUMMARY

This chapter has presented an original perfusion bioreactor designed and developed for 3D construct engineering. It has introduced the design and the flow mechanics of the system. This perfusion bioreactor system will be used to carry out experiments for 3D construct development as introduced in the next chapter.

6 CHAPTER SIX—IN VITRO CULTURE EXPERIMENT FOR 3D CONSTRUCT DEVELOPMENT USING PERFUSION BIOREACTOR

This chapter presents two culture experiments conducted using the developed perfusion bioreactor. The experiments aim to further examine and understand 3D tissue construct development under perfusion culture. The aspects of cell retention and cell proliferation, which are very important indicators for the status of 3D tissue assembly, are looked into. Experiment 1 investigates the aspect of cell retention and Experiment 2 studies the aspect of cell proliferation.

6.1 EXPERIMENT DESIGN

Figure 6-1 shows the general process flow of the *in vitro* experiments. In the preparation stage, highly porous 3D collagen scaffolds were fabricated through freeze-drying technique. Collagen was used as the scaffold material because it has very good biocompatibility and porous collagen sponges have been commonly used as tissue engineering scaffolds [170]. Concurrently, the frozen cells were resuscitated and expanded in culture flasks to obtain the required quantity of cells. The cells were then counted to prepare cell seeding solutions of desired cell density. In the experimental stage, appropriate amounts of cells were seeded onto the prepared scaffolds. The cell seeded scaffolds were then investigated for cell retention under perfusion (Experiment 1) or cell proliferation under different perfusion rates (Experiment 2). At the end of the experiments, constructs were stained with MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) staining to check the cell number and observed under scanning electron microscope (SEM) to study the cell and construct morphology.

Figure 6-1: General process flow for *in vitro* perfusion culture experiments

6.2 EXPERIMENT PREPARATION

As shown in Figure 6-1, the experiment preparation stage involved scaffold fabrication and cell preparation. This section introduces the materials, reagents and protocols used in the processes.

6.2.1 MATERIALS AND REAGENTS

The materials and reagents used in the *in vitro* study included:

- Collagen Powder: Type 1 collagen derived from bovine Achilles tendon (Invitrogen, C9879-25G)
- Acetic acid: 0.05M acetic acid used as solvent for the collagen powder
- Complete culture medium: 90% Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, 11995065), 10% Fetal Bovine Serum (FBS) (Invitrogen, 10500064) and 0.01% Penicillin-Streptomycin (Invitrogen, 15140122)
- 0.5% Trysin-Ethylenediaminetetraacetic acid (EDTA) solution (Invitrogen, 15400054)
- 0.4% Trypan blue stain (Invitrogen, 15250061)
- Powder-form phosphate buffered saline (PBS) (Invitrogen, P3813)
- Recovery™ Cell Culture Freezing Medium(Invitrogen, 12648010)
- Human dermal fibroblasts (HDF) (Lonza, CC-2509-SP)
- MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Invitrogen, M6494)
- 10% sodium dodecyl sulfate (SDS) solution (Invitrogen, 15553027)
- DMEM without phenol red (Invitrogen, 21063029)
- Glutaraldehyde solution (Sigma-Aldrich, 000060)

6.2.2 SCAFFOLD FABRICATION

The collagen scaffolds were constructed using freeze-drying method. In brief, suspension of collagen solution was firstly molded in wells of 24-well plates. The plates were then frozen to create continuous networks of ice crystal surrounding collagen fibers. The ice crystals were afterwards sublimated through freeze-drying

process, leading to formation of a highly porous collagen scaffold with pore structure mimicking that of the ice crystals. While the formation and structure of ice crystals were essentially controlled by the local thermal condition, uniform pore structure of the scaffolds was obtained by engaging uniform freezing process for the scaffold body.

The procedures were as follows:

- i. Dissolve 1 gram of collagen powder in 100 ml 0.05M acetic acid to obtain 1% (g/ml) of collagen solution.
- ii. Stir the solution for 5 hours under room temperature to achieve solution homogeneity.
- iii. Aliquot the solution into wells of 24-well plates, approximately 2 ml per well.
- iv. Freeze the plates at -80°C freezer for 2 days.
- v. Freeze dry the frozen plates for 2 days.
- vi. Cut away the top and bottom layers of the scaffolds to obtain 5 mm thick scaffolds.
- vii. Keep the prepared scaffolds in the fungus-free desiccator.
- viii. Just before the cell seeding, take out scaffolds from the desiccators and sterilize them in 70% ethanol for 30 minutes. Then wash them with autoclaved deionized water for 3 times and 5 minutes each. Subsequently, rinse them with sterile PBS solution and prepare for seeding.

6.2.3 CELL CULTURE AND MAINTENANCE

Good cell culture practice is essential for normal cell growth and proliferation.

6.2.3.1 Thawing and resuscitation of cells

At the beginning of culture process, cells were retrieved from the liquid nitrogen storage tank and thawed to 37°C. Experimental procedures to thaw and resuscitate the cells were as follows:

- i. Thaw one frozen vial of cells in 37°C water bath.
- ii. Pipette the cell solution from the vial into one T75 culture flask¹.
- iii. Add 20 ml culture medium into the flask and resuspend the cell solution.
- iv. Transfer 10 ml of the cell solutions into another T75 culture flask.
- v. Transfer the flasks into a CO₂ incubator for culture.

6.2.3.2 Cell passaging

Cells were subcultured at 70%--80% confluency to ensure they grow in appropriate densities. The cell passaging procedures were as follows:

- i. Check the T75 culture flask under converted light microscope for confluency and inspect any abnormal cell growth condition such as contamination and cell death.
- ii. If cells grow normally and are 70%--80% confluent, remove and discard the medium in the flask.
- iii. Wash the cell layer with 5 ml PBS solution for each culture flask and remove the supernatant.
- iv. Add 5 ml of 0.05% Trypsin-EDTA solutions into each flask.
- v. Incubate the flasks for 2—5 minutes for cell detachment.
- vi. When around 90% of the cells are detached from the flask substrate (inspected under light microscope), add 5 ml culture medium into the flask to

¹ Culture flask with 75 cm² culture area

neutralize the solution.

- vii. Transfer the liquid in the flasks to a 15 ml centrifuge tube and centrifuge at 1500 rpm for 3.5 minutes.
- viii. Remove and discard the supernatant from the centrifuge tube.
- ix. Add 10 ml fresh culture medium to the centrifuge tube. Resuspend to obtain homogeneous cell solution.
- x. Evenly aliquot the 10 ml cell solution into 2 new T75 flasks.
- xi. Add 10 ml fresh medium to each new T75 flask and resuspend to obtain even cell solution.
- xii. Transfer the culture flasks into the CO₂ incubator.

6.2.3.3 Cryopreservation

Extra cells were cryopreserved for future use. The cryopreservation procedures were as follows:

- i. Repeat steps i to viii in Section 6.2.3.2.
- ii. Add proper amounts of freezing medium into the centrifuged cells and resuspend to obtain homogeneous cell solution with density around 10⁶ cells/ml.
- iii. Transfer the cell solution into freezing vials, 1 ml per vial.
- iv. Label the vials.
- v. Keep the vials in -80°C freezer for 24 hours and then transfer them into liquid nitrogen tank for long-term storage.

6.3 CHARACTERIZATION METHODS

At the end of the experiments, 3D constructs were characterized for the cell population and cell morphology through MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-

diphenyltetrazolium bromide) staining and scanning electron microscope (SEM) observations.

6.3.1 *MTT STAINING*

The determination of cellular proliferation and viability are key areas in cellular biological studies. MTT is one of the most commonly used assays for the purpose. The MTT stain is reduced to purple formazan in the mitochondria of living cells. The purple MTT formazan crystals are then yielded by mitochondrial dehydrogenases which cleave the tetrazolium ring. The resulting purple solution is spectrophotometrically measured at 570nm wavelength. The absorbance reading indicates the formazan density, which is a direct indication of the quantity of stained viable cells.

The procedures for MTT reading were as follows:

- i. Prepare 5 mg/ml MTT solution by dissolving MTT powder in sterile PBS solution.
- ii. Transfer the scaffolds into the wells of 24-well plate, one scaffold per well.
- iii. Rinse the scaffold with PBS gently and remove the supernatant.
- iv. Add 500 μ l DMEM without phenol red to each well.
- v. Add 50 μ l MTT solution to each well. Wrap the plate with aluminum foil to prevent the plate from exposure to light.
- vi. Agitate the plate on plate shaker gently for 5 minutes for even staining of the scaffold.
- vii. Incubate the plate for 3 hours.
- viii. Add 500 μ l SDS solution to each well and mix the solution thoroughly.
- ix. Incubate the plate for 18 hours.

- x. Stir the solution in the well thoroughly to make it even. Transfer the solution into wells of 96-microwell plate, 100 μ l per well.
- xi. Put the microwell plate into microplate reader and read the absorbance at 570 nm.

To understand the relationship between the absorbance reading value and seeded cell number, MTT readings should be calibrated beforehand. For this purpose, a series of seeding volumes: 0 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1 ml, with the same seeding cell density of 5.6×10^5 cells/ml were used to seed on a set of scaffold groups. Each scaffold group had 4 samples to check result repeatability except the 0 ml group, for which only 2 samples were used as reading results were usually quite consist in the group (based on past observations of the research). The averaged absorbance readings for individual scaffold groups were shown in Table 6-1.

Table 6-1: MTT reading for calibration

Seeding density: 5.6×10^5 cells/ml						
Seeding volume		0 ml	0.4 ml	0.6 ml	0.8 ml	1 ml
Seeding cell number		0	2.58×10^5	3.36×10^5	4.48×10^5	5.60×10^5
Absorbance reading	Scaffold 1	0.097	0.2372	0.2276	0.3084	0.3215
	Scaffold 2	0.0944	0.2024	0.2337	0.2599	0.3287
	Scaffold 3	N.A.	0.2426	0.2399	0.3068	0.3304
	Scaffold 4	N.A.	0.1681	0.2856	0.3428	0.4047
	Mean	0.0957	0.212575	0.2467	0.304475	0.346325
	After calibration with control	0	0.01169	0.1510	0.2087	0.2506
	Sta Dev	0.001838	0.03459	0.026415	0.034042	0.039107

The standard deviations for each scaffold group were calculated and listed as “Sta Dev” in Table 6-1. Their small values relative to the means indicated that the seeding efficiency for the scaffolds in the same seeding group was quite consistent. Based on the observed absorbance reading data, the MTT calibration curve was plotted and is shown in Figure 6-2.

Figure 6-2: MTT calibration curve

Based on Figure 6-2, the absorbance reading of scaffold is in liner relationship with the seeding cell number. It is noted that this calibration was purposely carried out using 3D scaffolds, which differed from the traditional 2D culture practice. This was to ensure the calibrated curve could be used for determining the cell number for 3D tissue construct culture in the work.

6.3.2 SEM OBSERVATION

SEM pictures were taken to study the morphology of cells and scaffolds after culture at micro-levels. The procedures were as follows:

- i. Transfer the scaffolds into wells of a new 24-well plate, one per well.
- ii. Fix the scaffolds in 2.5% glutaraldehyde solution for 2 hours.
- iii. Remove the fixation medium and wash the scaffolds with deionized water for 4 times, 10 minutes each.
- iv. Dehydrate the scaffolds with a series of ethanol in the following order:
 - 50% ethanol for 10 minutes, once
 - 70-75% ethanol for 10 minutes, once

90-95% ethanol for 10 minutes, once

100% ethanol for 10 minutes, twice

- v. Dry the dehydrated scaffolds with critical point dryer.
- vi. Cut the dried scaffold to display the scaffold top surface, bottom surface and axial cross section with razor blades. Mount the pieces onto the specimen stub.
- vii. Coat the pieces with gold.
- viii. Observe the coated pieces under SEM.

6.4 EXPERIMENT 1—PERFUSION CULTURE EFFECTS ON CELL RETENTION

Dynamic culture can affect 3D bioconstruct development from various aspects such as cell metabolism, cell population and extracellular matrix assembly [131, 171]. Among the different aspects, cell population is the basis of biological activities in the construct. It changes with cell proliferation and cell retention in 3D constructs under flow stimulation.

A large number of studies in literature have investigated the cell proliferation under dynamic culture in 3D constructs [172-175]. However these studies have largely ignored the aspect of cell retention, which could also largely influence the cell population in the construct. If dynamic flow causes significant cell loss thus low cell retention ratio, the total cell number would still be low even though flow may stimulate cell proliferation. To better understand cell population change in 3D construct development under dynamic culture, the flow effects on cell proliferation and cell retention should be differentiated. In particular, the relationship between cell retention and dynamic culture should be investigated and further clarified.

Previous studies on perfusion culture have usually cultured constructs for days to weeks [172-175]. The limitation of such studies is that the observed cell number in the construct is co-influenced by cell proliferation and cell loss. In comparison, in short culture durations (minutes to hours), cell number change can be directly linked to cell retention without concerns of cell proliferation. Thus, to study the flow effects on cell retention, short-term culture (minutes to hours) would be a better study model as compared to long-term culture.

This work is thus motivated to investigate the cell retention in 3D constructs under short-term perfusion culture. It is hypothesized that 3D constructs perfused under lower flow rates retain more cells as compared to that under higher flow rates. It is also hypothesized that the perfusion direction has no influences on cell retention. The knowledge obtained from this work helps tissue engineers to understand the flow effects on cell population change in 3D constructs during the initial periods of perfusion culture.

6.4.1 METHODOLOGY

Human dermal fibroblasts (HDFs) were used as the cell model in the study. Porous scaffolds with 5 mm thickness and 7 mm radius were fabricated from collagen as described in Section 6.2.2. Three groups of scaffolds were prepared. The microstructures of the scaffold were inspected under SEM.

4.6×10^5 HDFs were seeded onto each scaffold from the scaffold top surface by static seeding. Scaffolds were then incubated overnight for the cells to stabilize. On the next day, scaffolds in groups 1 and 2 were transferred into the perfusion bioreactor as described in Chapter 5 for perfusion. Group 1 scaffolds were perfused from the construct top surface (the cell seeded surface). Group 2 scaffolds were perfused

from the construct bottom surface (the unseeded surface). Group 1 and Group 2 were compared to investigate the effect of perfusion direction on cell retention in the constructs. Both groups contained 4 subgroups, which were perfused with different flow rates 2.3 ml/min, 3.6 ml/min, 4.9 ml/min and 6.1 ml/min respectively. The different subgroups were compared to investigate the impact of perfusion flow rate on cell retention. Each subgroup contained 4 samples, which were sequentially connected in the same flow circuit. The perfusion lasted until flow passed through all 4 samples, linking the perfusion duration with perfusion rate for each subgroup. The measured perfusion duration for 2.3, 3.6, 4.9, 6.1 ml/min perfusion subgroups were 6.6, 4.3, 3.2, 2.5 minutes respectively. Group 3 was the control group, which had 4 samples and did not undergo the perfusion process.

After the perfusion, scaffolds were stained with MTT and the MTT absorbance readings were taken at the wavelength of 570 nm.

All data are presented as means +/- standard error. Data sets were analyzed using two way ANOVA followed by Fisher's Least Significant Difference (LSD) Test. $P < 0.05$ was considered statistically significant. The analysis results are presented in detail in Appendix B.

6.4.2 RESULTS

6.4.2.1 Microstructure of scaffold

Figure 6-3 shows the scanning electron microscope (SEM) picture for the scaffold cross section. The scaffold had a highly porous microstructure, which was regular with pore diameters ranging from 50 to 100 μm . In addition, the pores were shown to be interconnected, which could facilitate mass exchange, flow penetration and cell proliferation and migration during 3D construct development [129-130].

Figure 6-3: Microstructure of the scaffold

6.4.2.2 Effect of perfusion rate

Figure 6-4 presents the MTT absorbance reading of 3D constructs after perfusion at different flow rates and directions.

Figure 6-4: Cell population in the construct after perfusion. Thin bars represent the standard error of the means ($n = 4$). Asterisk denotes a statistically significant difference.

Based on Figure 6-4, perfused constructs always held lower cell numbers compared to unperfused samples, indicating flow induced cell loss. The cell numbers in constructs perfused under 3.6 ml/min ($t=2.9574 > t_{\text{critical}}=2.042$) and 4.9 ml/min ($t=3.3506 > t_{\text{critical}}=2.042$) were significantly lower than that in the control (0 ml/min). The power of statistical analysis for comparison at both these two flow rates is higher than 0.85. The cell numbers in perfused samples under other perfusion rates were lower than the control but the differences were statistically insignificant. Thus, different flow rates correspond to different cell retention ratios in the perfused constructs.

At the low flow rate region (lower than 4.9 ml/min), cell number in the constructs decreased with flow rate. This was likely because higher flow rate induced higher flow shear stress, which caused more cell detachment from the construct. In comparison, at higher flow rate region (higher than 4.9 ml/min), cell number in the construct increased with flow rate, which was unexpected. A possible reason for this could be linked to perfusion duration. As the flow rate increased, the perfusion duration decreased. Though increasing flow in this flow region could result in higher flow shear to induce more cell loss, the perfusion duration might be too short for cells to detach. It was likely that cells in the construct were different in their detachment strength and those with stronger detachment strength required a longer perfusion time to detach from the construct.

6.4.2.3 Effect of perfusion direction

As seen from Figure 6-4, constructs perfused from top surface (cell seeded) in general retained more cells than that perfused from bottom surface (unseeded) except the case of 3.6 ml/min perfusion. The 3.6 ml/min perfusion was re-examined in a new set of experiments and similar results were obtained. Thus, cell retention in

3D constructs under perfusion appeared to be influenced by the perfusion direction, with co-influences from perfusion rates.

Statistical analysis indicated that there were no significant influences of perfusion direction to cell retention in the construct ($F=1.0246 < F_{\text{critical}}=4.1709$, $p=0.3195$). The minor differences caused by perfusion direction on cell retention as observed in Figure 6-4 should be linked to the cell distribution in the constructs. In the study, cells were seeded from construct top surface by static seeding, which is typically concerned with cell congregation near the scaffold top surface region. When scaffolds were perfused from the top (seeded) surface, two possible events could occur. Firstly, the interstitial flow induced by perfusion could bring cells from the construct top surface into deeper regions of the construct. This event could assist cell retention in the construct. Secondly, the flow could directly flush cells away from the construct top surface into the surrounding medium. This event would lead to cell loss and reduced cell retention ratio. It was also possible that some components of the scaffold could be broken away from the main scaffold body under the flow shear, leading to the loss of cells that had originally adhered to the detached components. In comparison, when scaffolds were perfused from the bottom (unseeded) surface, cells at the construct top surface region were sheltered from direct flow shock by the scaffold materials at the construct bottom region. As a result, cells were protected from being flushed away from the construct's top surface and more cells were retained. However, the interstitial flow direction in the construct was reversed, which could on the other hand bring cells out of the construct into surrounding medium and lead to cell loss.

It is possible that the cell retention in constructs under perfusion was a function of the counter effects of the interstitial flow in the construct and the external flow on

construct surfaces. Under 3.6 ml/min perfusion, the cell number in constructs perfused from top surface was lower compared to that in constructs perfused from bottom surface. Such an observation could be because more cells were flushed away from the construct's top surface in the former compared to that were washed out of the constructs by interstitial flow in the latter.

6.4.3 *DISCUSSION*

To utilize cells for 3D construct development, cells should be retained as many as possible in the culture process. The cell retention in 3D constructs under dynamic culture can be affected by various factors such as chemical properties of the scaffold [176-177], the perfusion duration and flow rate [178-179]. This work has investigated the factor of perfusion culture from the aspects of flow rate and perfusion direction. Under long-term (days to weeks) perfusion culture, both cell loss and cell proliferation are possible in perfused constructs, which makes it difficult to analyze the cell retention ratio based on the total cell number in the construct. Thus the perfusion period in this work was purposely designed to be short (minutes to hours) to avoid the concern for cell proliferation induced cell number change. The short perfusion duration represents the initial stage of perfusion culture.

MTT staining results indicated reduced cell number in perfused constructs compared to unperfused samples. While MTT stains the mitochondria of live cells, the lower cell number under perfusion could be caused by either cell detachment or cell death. However, as perfusion duration in the study was relatively short and constructs were stained just after the perfusion, flow induced cell detachment, rather than cell death, should be the possible cause for the cell number reduction.

By assuming parabolic flow of media with viscosity μ of 0.01g/cm-s through cylindrical pores with diameter d of 80 μ m, the shear stress in the construct was estimated as [175]:

$$\tau = 8 \mu Q / (\pi L^2 d)$$

Q is the volume flow rate in the construct; L is the construct radius of 0.7cm. The calculated shear stresses in constructs under 2.3, 3.6, 4.9, 6.1 ml/min perfusion were 0.25, 0.39, 0.53, 0.65 dyn/cm² respectively. However, the estimated shear stress levels in this work cannot be compared with literature as there are no reference values for fibroblast attachment strength in 3D collagen matrix from previous studies. Nevertheless, it is expected that the cell adhesion strength varies from cell to cell depending on the local scaffold microstructure and individual differences of cells [180]. It would take longer for cells with higher adhesion strength to detach from the construct.

The observed cell retention dependence on flow rate was as expected. When the shear force exceeds cell adhesion force, cells detach from the scaffold. At low flow rate region (small than 4.9 ml/min), retained cell number in the construct decreased when the flow rate increased. It is likely because more and more cells with higher adhesion strength were washed away from the construct with the increasing flow shear in the process. When flow rates exceeded 4.9 ml/min, cell retention ratio increased with flow rates in the constructs, which was likely due to the shorter perfusion period. In the experiments, the higher the perfusion rate, the shorter the perfusion duration. It was possible that if given longer perfusion time, more cells would have rounded up and detached from the construct at the same flow rates in the high flow region (greater than 4.9 ml/min). This inference was in agreement

with previous studies that cell detachment under fluid flow was perfusion duration dependent under the same flow shear stress [179].

ANOVA analysis revealed that there was no significant difference to cell retention due to change of perfusion directions. In addition, statistics showed that there was no significant interaction effects between perfusion rates and directions. However, judging from the reading in general, constructs perfused from the cell seeded surface retained slightly more cells compared to that perfused from the unseeded surface. The influence of perfusion direction on cell retention in the construct is suspected to be due to the uneven cell distribution in the construct. Static seeding deposited most cells on the construct seeding side. As a result, perfusion from the seeded side of the construct could assist cell infiltration from the construct surface into the inner construct region with interstitial flow, leading to higher cell retention ratio compared to perfusion from the unseeded side. On the other hand, when constructs were perfused from the unseeded side, direct contact between construct seeded surface and entering flow was avoided. This might have helped protect cells on the construct top surface from being flushed away by the external flow.

The study shows that cell number in perfused constructs could be reduced significantly even for short perfusion durations due to cell detachment, for which the flow rate and direction could be co-influencing factors. It is noted that this study was based on a short perfusion period which represents the initial stage of perfusion culture. It is possible that further cell loss from the construct could occur during the later stages of the culture. Such flow induced cell loss would make it difficult to analyze the perfusion culture outcome based on the initial system setup. For example, the number of cells in the construct for proliferation is no longer the known seeding number once the culture starts and analysis of cell growth rate

would be problematic. To achieve higher cell retention ratio and reduce flow induced cell detachment, the flow rate could be lowered to reduce the flow shear stress. However, this method might lead to a compromise on the flow-promoted mass exchange.

6.4.4 CONCLUSION FOR EXPERIMENT 1

This work has studied the impact of short-term perfusion on cell population in 3D constructs. In the given experimental conditions, flow perfusion led to reduced cell number in the scaffolds due to the flow induced cell detachment. Perfusion rates instead of perfusion direction brought statistically significant influences to the cell retention ratio. While cell loss could distort the culture process, it should be considered for analyzing the dynamic culture outcome. In the future, engineering methods can be explored to vary other culture parameters (e.g., scaffold designs [181-188], perfusion durations) to further examine cell retention in 3D constructs under perfusion culture, together with the help of computational fluid modeling [189]. The findings in this work offer tissue engineers a hint about the possible consequences of perfusion culture on cell retention in 3D construct development.

6.5 EXPERIMENT 2—PERFUSION CULTURE EFFECTS ON CELL PROLIFERATION

Perfusion culture provides an improved growth environment for cells especially at the inner regions of 3D constructs and promotes better cell growth compared to static culture [190-192]. This experiment looks into the influences of perfusion rate and duration on cell proliferation. It is hypothesized that higher flow rates promote higher number of cell growth and cell proliferation rate in the construct decreases with time due to the increasing cell packing density.

6.5.1 METHODOLOGY

The in-house developed bioreactor as described in Chapter 5 was used for the perfusion study. Three groups of scaffolds were fabricated and prepared. They were seeded with human dermal fibroblasts of passage 7. The seeded scaffolds were incubated for 24 hours in static culture to let cells to stabilize. Afterwards, the 3 groups of constructs were individually perfused under different flow rates: 1 ml/min, 2 ml/min and 3 ml/min. The flow rates were selected to bring observable difference to cell growth based on comparable culture studies in literature [52]. The culture process lasted for 7 days. As the cells had been observed with healthy doubling until passage 17, the cells were expected to grow without aging concerns during the 7 day culture. The scaffolds were examined with cell numbers through MTT staining right after seeding and after 1, 3, 5 and 7 day perfusion (n=4). The scaffolds perfused under 1 ml/min and 2 ml/min were also observed under SEM to check the cell distribution and morphology on the perfused scaffold. The scaffolds perfused under 3 ml/min were not examined under SEM as SEM observations for scaffolds under the two lower flow rates were sufficient for studying the cell morphology under different perfusion rates and duration. Static culture was conducted as control for all perfusion groups.

All the data was presented in mean +/- standard deviation. Two-tail t-test was used for analyzing the data between dynamic and static culture groups at the same time points. $p < 0.05$ is considered as significant.

6.5.2 RESULTS

6.5.2.1 Cell growth under 1 ml/min perfusion

Table 6-2 presents the MTT absorbance readings for scaffolds perfused under 1 ml/min. The standard deviation for each scaffold group was calculated and presented in the table as “Sta Dev”. With reference to the MTT reading calibration curve in Section 6.3.1, the initial cell number for scaffolds in 1 ml/min study group was 7×10^5 . Figure 6-5 plots the cell number change with culture duration in the construct.

Table 6-2: MTT absorbance for scaffolds under 1 ml/min perfusion

Days	Dynamic		Static	
	Average	Sta Dev	Average	Sta Dev
0	0.3637	0.0548	0.3637	0.0548
1	0.4026	0.0708	0.3696	0.0768
3	0.5546	0.0630	0.4337	0.0686
5	0.5354	0.0557	0.4011	0.0624
7	0.535	0.030026	N.A.	N.A.

Figure 6-5: Cell number change with time for constructs perfused under 1 ml/min. Thin bars represent the standard deviation.

Data at day 7 under static culture was missing in the work. This was because the MTT staining for the dynamically cultured samples was observed to be very light and the MTT reading indicated extremely low cell numbers in the samples (comparable to that of unseeded scaffold). Such results were considered abnormal and likely caused by contamination. A repetition of the experiment was not considered as the culture conditions between different batches were likely to vary leading to incomparable culture results.

Results show that under 1 ml/min perfusion culture, cell number increased in the first 4 days. During this period, cell number increasing rate was higher after day 1 compared to that before day 1. Such growth rate agreed with typical exponential proliferation of cells.

After day 4, cell number in the perfused construct remained relatively constant. The possible explanations could be firstly MTT staining result was dependent on the mitochondrial activity of intact viable cells, which however were impaired due to the shear flow or dense cell accumulation in the studied samples. In addition, after day 4 cells could have actually stopped proliferation as local cell density level reached too high leading to contact inhibition. High local cell number could be resulted from uneven cell seeding, which is a typical problem for seeding 3D constructs statically, or inhomogeneous cell proliferation due to uneven flow stimulations.

The time interval of two days for data sampling was determined with reference to cell growth rate (doubling in two days in T75 flask) and the experimental cost. It is worth pointing out that due to the limitation of time points, the interpolated cell number could vary from the actual cell number in the construct. The trend line generated from the experimental data thus is more suitable to be used for studying

the general trend of cell population change rather than accessing the exact cell number at particular time points.

Cell growth under static culture followed a similar trend as that under 1 ml/min perfusion culture. However, the cell number in static culture was always lower than that in dynamic culture, indicating that cell proliferation was promoted under the perfusion culture environment. Two-tail t-test shows that there were significant differences between the static and dynamic culture groups on day 3 ($p=0.0211$) and day 5 ($p=0.0098$).

Cell morphology and distribution in the construct were examined under SEM at different time points during perfusion culture. Three surfaces of the constructs: the unseeded/bottom surface, the axial cross section and the seeded/top surface as shown in Figure 6-6 were inspected.

Figure 6-6: Scaffold surfaces for SEM observation

Figure 6-7 compares the SEM images for constructs after 1 day of 1 ml/min perfusion culture and static culture.

Figure 6-7: SEM observation for constructs after 1 day of culture (1 ml/min). (a1) is the unseeded surface, (b1) is the cross section and (c1) is the seeded surface of construct under perfusion. (a2), (b2) and (c2) are the corresponding surfaces of construct under static culture. Arrow heads indicate cells. Red curves mark the shapes of some cells for illustration purpose.

Figure 6-7 shows that cell distributed rather unevenly on the scaffold. For both dynamic and static culture samples, many more cells accumulated on the cell seeded surface compared to that on the unseeded surface or cross section. This could be largely attributed to the initial uneven cell seeding of the scaffold. Cross section view shows that very few cells managed to migrate into the inner scaffold region. In general, most of cells grew on the top surface of scaffolds and there were relatively more cells on the dynamically cultured scaffolds.

Figure 6-8 presents the SEM observation for scaffolds after 3 days of culture.

Figure 6-8: SEM observation for constructs after 3 days of culture (1 ml/min). (a1), (b1) and (c1) are the respective unseeded bottom surface, cross section and cell seeded surface of perfused scaffold. (a2), (b2) and (c2) are the respective surfaces for scaffolds under static culture. Arrow heads indicate cells. Red curves mark the shapes of some cells for illustration purpose.

On day 3, uneven cell distribution similar to that on day 1 culture was observed for both statically and dynamically cultured scaffolds. However there were more cells on the scaffolds for both groups compared to that after 1 day culture, which was in agreement with the MTT reading. Distinctively, in the perfused scaffold, the cell seeded side was covered with very densely packed cells and cells appeared highly elongated and aligned. In comparison, cells in scaffolds under static culture were less dense and did not show any regular orientations or elongated morphologies. The cell alignment on perfused construct was likely resulted from flow stimulation.

Figure 6-9 presents the SEM observation for scaffolds after 5 days of culture.

Figure 6-9: SEM observation for constructs after 5 days of culture (1 ml/min). (a1), (b1) and (c1) are the respective unseeded bottom surface, cross section and cell seeded surface of the perfused scaffold. (a2), (b2) and (c2) are the respective surfaces for scaffolds under static culture. Arrow heads indicate cells. Red curves mark the shapes of some cells for illustration purpose.

On day 5, cells on the seeding surface of perfused scaffolds displayed a highly elongated morphology and were highly oriented while that on statically cultured scaffolds had no such morphology features. Compared to the condition after 3 days of culture, there were more cells on the outer surfaces (both unseeded and seeded surfaces) of perfused scaffolds.

Figure 6-10 presents the SEM observation for scaffolds after 7 days of perfusion.

Figure 6-10: SEM observation for constructs after 7 days of perfusion (1 ml/min). (a), (b) and (c) are the respective unseeded bottom surface, cross section and cell seeded surface of the perfused scaffold. Arrow heads indicate cells. Red curves mark the shapes of some cells for illustration purpose.

On the cell seeding surface of scaffolds after 7 day perfusion, cells were so densely packed that almost no pores could be observed on the scaffold surface. On the

opposite (unseeded) surface of the scaffold, cells had also formed a dense layer. In contrast, the cross section was observed with barely any cells. It is speculated that the dense cell layer on the outer surfaces of the scaffold acted as a strong barrier for mass exchange between outer and inner scaffold regions as well as cell infiltration into the scaffold. The hindrance led to inferior cell growth environment and low cell number in the inner scaffold region. In addition, the high local cell packing density on scaffold outer surface could impair cell metabolism and proliferation due to contact inhibition, which could be linked to the stagnant cell population in the construct at the later stage of culture.

It is worth pointing out that the distribution of cells in the construct was observed to be inhomogeneous under SEM. SEM images such as those in Figures 6-7 to 6-9 only presented the observation on local points of the construct surface. They thus should not be used to judge the overall cell population in the construct. Instead, they are preferred to be used for examining the morphology and distribution of the cells in general. To compare cell numbers in different culture groups, MTT reading should be referred to.

6.5.2.2 Cell growth under 2 ml/min perfusion

Table 6-3 presents the MTT absorbance of scaffolds at different culture time points under 2 ml/min perfusion. Static culture results were used as control. Referring to the MTT calibration curve, the initial cell number at the starting of the culture was 8.7×10^5 . Figure 6-11 presents the corresponding plot for cell number change in the construct with culture time.

Table 6-3: MTT absorbance of scaffolds under 2 ml/min perfusion

Days	Dynamic		Static	
	Average	Sta Dev	Average	Sta Dev
0	0.4357	0.0608	0.4357	0.0608
1	0.4204	0.0461	0.3837	0.0338
3	0.4851	0.0741	0.3989	0.0572
5	0.4738	0.0676	0.4602	0.0367
7	0.4261	0.0100	0.4446	0.0310

Figure 6-11: Cell growth with culture time under 2 ml/min perfusion. Thin bars indicate standard deviation.

For both dynamic (2 ml/min perfusion) and static culture, cell number decreased at day 1, then gradually increased till day 5 and afterwards remained relatively constant till day 7. Compared to that under dynamic culture, scaffolds in the static control groups contained fewer cells (statistically insignificant) during most of the culture time, indicating the relative advantage of perfusion culture for promoting cell proliferation. However, static group appeared to proliferate faster after day 3 and reached a similar cell number as the dynamic group at day 5. This showed that 2 ml/min perfusion did not always lead to better cell proliferation, possibly due to local cell densities in the construct and cell adaptation to the flow.

In general, cell growth profile from day 1 to 7 under 2 ml/min perfusion was similar to that under 1 ml/min perfusion culture, except that cell number had an initial

decrease instead of increase before day 1 under 2 ml/min perfusion. This difference could be due to different cell seeding number or the different perfusion rates for the two cases.

The SEM observation for cell distribution and morphology under 2 ml/min perfusion are presented in Figures 6-12 to 6-15. SEM images for static culture group are also provided for comparison.

Figure 6-13 presents the SEM observation for constructs after 1 day of culture.

Figure 6-12: SEM observation for constructs after 1 day of culture (2 ml/min). (a1), (b1) and (c1) are the respective unseeded bottom surface, cross section and cell seeded surface of perfused scaffold (2 ml/min). (a2), (b2) and (c2) are the respective surfaces for scaffolds under static culture. Arrow heads indicate cells. Red curves mark the shapes of some cells for illustration purpose.

On day 1, under 2 ml/min perfusion, cells grew at a much high density on the seeding side of the scaffold compared to that on the unseeded side and cross section, similar to the observation under 1 ml/min perfusion. There were more cells on the perfused scaffolds than on statically cultured scaffolds, in agreement with MTT absorbance readings. On the seeded surface of the perfused samples, cell alignment was already observable, earlier than that under 1 ml/min perfusion, indicating that a higher flow rate might have led to faster cell alignment in perfusion culture.

Figure 6-13 presents the SEM observation for constructs after 3 days of culture.

Figure 6-13: SEM observation for constructs after 3 days of culture (2 ml/min). (a1), (b1) and (c1) are the respective unseeded bottom surface, cross section and cell seeded surface of perfused scaffold. (a2), (b2) and (c2) are the respective surfaces for scaffolds under static culture. Arrow heads indicate cells. Red curves mark the shapes of some cells for illustration purpose.

On day 3, on the seeded surface of the perfused (2 ml/min) scaffolds, a very dense layer of cells was observed. These cells were further aligned compared to that after day 1 perfusion. On the unseeded surface, cells remained few similar to that on day 1. Hardly any cells can be seen on the scaffold cross section, indicating that cells barely migrated into the scaffold. The uneven cell distribution should be linked to initial uneven cell seeding and the blockage of scaffold and cellular materials on the outer surface of the scaffold. Compared to that on the dynamic culture group, fewer cells were observed on the static culture group, which agreed with the MTT reading. In distinct contrast to the aligned cell orientation in the dynamic culture group, cells on the static culture groups oriented randomly.

Figure 6-14 presents the SEM observation for scaffold sections after 5 days of perfusion (2 ml/min).

Figure 6-14: SEM observation for constructs after 5 days of culture (2 ml/min). (a1), (b1) and (c1) are the respective unseeded bottom surface, cross section and cell seeded surface of perfused scaffold. (a2), (b2) and (c2) are the respective surfaces for scaffolds under static culture. Arrow heads indicate cells. Red curves mark the shapes of some cells for illustration purpose.

On day 5, for both dynamic and static culture samples, very dense cell layers were observed on the cell seeding surface with slightly more cells on the dynamic culture samples. Barely any cells were found on the cross section indicating extremely uneven cell distribution in the scaffolds. Compared to day 3 samples, more cells were developed on the unseeded surface for both culture groups, indicating local cell proliferation. In general, compared to day 3 culture, there were distinctively more cells in the static culture group, but not for dynamic culture group, which agreed well with the MTT reading.

Figure 6-15 presents the SEM observation for construct sections under 2 ml/min perfusion on day 7.

Figure 6-15: SEM observation for constructs after 7 days of culture (2 ml/min). (a1), (b1) and (c1) are the respective unseeded bottom surface, cross section and cell seeded surface of perfused scaffold. (a2), (b2) and (c2) are the respective surfaces for scaffolds under static culture.

On day 7, the outer layers of scaffolds under both dynamic and static culture were covered with dense cell layers. As a result, almost no distinguishable pores could be found on scaffold surface, which was expected to strongly hinder the cell infiltration into scaffolds. It was thus no surprise to observe no cells on the cross section of the scaffold.

While the MTT reading showed that scaffolds on day 7 culture perfusion had slightly lower absorbance values than that from day 5 culture, SEM image revealed more cells on the outer surfaces of perfused scaffolds on day 7. The disagreement between the MTT reading and the SEM observation could be due to uneven cell distribution in the constructs.

6.5.2.3 Cell growth under 3 ml/min perfusion

Table 6-4 presents the MTT absorbance readings of scaffolds under 3 ml/min perfusion and static culture. Referring to the MTT calibration curve, the initial cell number in the constructs was 9.8×10^5 . Figure 6-16 plots the cell number change with time under the 3 ml/min perfusion.

Table 6-4: MTT absorbance of scaffolds under 3 ml/min perfusion

Days	Dynamic		Static	
	Average	Sta Dev	Average	Sta Dev
0	0.4930	0.0306	0.4930	0.0306
3	0.4992	0.0347	0.4862	0.0055
5	0.4106	0.0210	0.3795	0.0491
7	0.4311	0.0308	0.4628	0.0026

Figure 6-16: Cell proliferation with culture time under 3 ml/min perfusion

Based on MTT readings, cell number decreased after day 3 then slightly increased after day 5 for both dynamic (3 ml/min) and static culture. The decrease of cell number between day 3 and day 5 was unexpected. As both dynamic and static culture samples had similar decrease pattern, the decrease appeared to be resulted from reasons other than the effects of high flow rate. This could be related to the high cell density. The difference between the readings for dynamic and static culture groups during the entire culture process was very small. One possible reason could be the high initial seeding density, which consumed large amounts of nutrients at the earlier culture period and resulted in an inadequate nutritious environment for later cell growth. In addition, the high initial cell number in the construct could have covered a big portion of the outer surfaces of the construct, limiting the impact of flow to only the cells at the outermost layers of the constructs. Moreover, it was possible that the high flow rate had caused cell detachment which lowered the cell number in the construct in general.

6.5.3 DISCUSSION

6.5.3.1 Cell growth is perfusion rate dependent

Comparing the dynamic culture groups with static control for all the 3 perfusion rates, dynamic culture in general helped improve cell proliferation and total cell number in the scaffold. The flow also induced cell alignment. The higher the flow rate, the faster the cells were aligned.

Comparing perfusion culture results under different flow rates, it appeared that flow rate and cell proliferation were reversely related. The benefits from perfusion for cell proliferation diminished fast with the flow rate. Under 2 ml/min flow, cell proliferation rate was much slower than that under 1 ml/min flow. Under 3 ml/min perfusion, the improvement of cell proliferation became minor compared to static control.

The influence of flow rate on cell growth should be firstly related to the changed shear stress level and mass exchange. At higher flow rates, the shear stress level on the construct surface is larger. The shear stresses for the dynamic culture under 1, 2, 3 ml/min were estimated to be 0.10, 0.23, 0.34 dyn/cm² [175]. Shear stress at the physiological level promotes cell function but may become detrimental at excessive levels. However, there were no comparable studies in the literature for shear stress effects on fibroblast growth in 3D collagen scaffolds. Nevertheless, the observations at the shear stress levels in the work provided good references for analyzing perfusion experiments of similar setups in the future.

Another possible contribution to the observed difference in cell population under different perfusion conditions could be the different mass exchange under respective perfusion rates. As modeled in Chapter 4, the mass exchange under

perfusion should have been improved with the flow rate. However, experimental assessment of the mass exchange especially at the inner scaffold regions in the experiment was impractical with the available resources. In the experiment, the effect of the improved mass exchange was coupled with that of the shear stress. In the future, decoupling the effects of mass exchange and shear stress should be attempted to clarify the individual effects under given flow rates.

6.5.3.2 Under the same flow rate, cell growth rate changes with time

Under 1 ml/min and 2 ml/min perfusion, the cell number in the construct changed with time in a similar way. This showed that within the flow range 1 to 2 ml/min, the perfusion culture imposed regular stimulation effects for 3D cell growth. In the flow range, cell growth rate initially increased with time then slowed down and stabilized at the later stage of culture. The change in cell growth rate could be because increasing cell density caused contact inhibition and inhibited further cell proliferation. The change could also be linked to the gradually depleting nutrient and accumulating metabolic waste in the medium, which had a direct impact on cellular events. The observations under 1 ml/min and 2 ml/min perfusion confirmed the initial hypothesis of the work that cell proliferation rate in the cultured construct decreased with time.

Under 3 ml/min perfusion, cell growth also changed with time but in a different profile. Cell number remained relatively constant in the first 3 days of culture and decreased after day 3. The phenomenon might be related to the high initial cell number at the start of the culture. High densities of cells could have consumed large amounts of nutrients, resulting in a nutritious medium that is inferior for local cell growth and proliferation at the later culture time.

6.5.3.3 Uneven construct development and cell distribution

Uneven cell distribution was observed in constructs under both dynamic and static culture. For all the cultured samples, cells hardly migrated into the inner scaffold region. This greatly constrained the growth space of cells in the scaffold and imposed a serious challenge for developing thick tissues. The uneven cell distribution and growth in the study could be readily related to uneven cell seeding. Though scaffolds were highly porous with interconnected pores, the infiltration of cells by solely gravity force in static cell seeding was shown quite inefficient. Cells mostly aggregated on the seeded surface of the scaffold and formed a dense layer covering the scaffold. The formed cell layer further prevented the outside cells from infiltrating into the scaffold. Eventually, the seeding surface of the scaffold had very high cell packing densities, but the cell distribution in the construct on the whole was highly uneven. To improve cell distribution in the construct, more efficient seeding methods should be utilized in the future.

6.5.3.4 Comparison with CFD model

Under flow rate 2 ml/min, the average flow rate in the scaffold by assuming even flow through the scaffold is:

$$\frac{\text{volume flowrate}}{\text{scaffold cross section area}} = \frac{2\text{ml/min}}{\pi*(0.7\text{cm})^2} = 0.02 \text{ cm/s.}$$

The cell number growth profile under 0.02 cm/s perfusion observed in experiments has been presented in Figure 6-11 earlier.

The cell number growth profile under 0.02 cm/s perfusion based on the model from Chapter 4 is presented in Figure 6-17. The model considers the effects of both shear stress and nutrient to cell growth.

Figure 6-17: Evolution of cell volume ratio in construct under 0.02 cm/s perfusion based on shear sensitive analysis (nondimensionalized)

Comparison between Figure 6-11 and 6-17 shows dramatic difference between the experimental and the simulation results. In the experiment, cell number in the construct stabilized at around day 6. However, in the simulation, cell number increased steadily in the first 25 days. The difference between experimental and simulation results could be possibly due to the following reasons:

- 1) Though the flow rate in the experiment was estimated to be the same as that in simulation, the actual flow in the bioreactor may be varied due to flow channeling. This is especially true with long term culture of fibroblasts which apply contractile forces to the construct during culture.
- 2) Cells are distributed quite unevenly in constructs in the experiment with the majority concentrated on the scaffold seeding side. In the simulation study,

even cell distribution in the construct is assumed.

- 3) There are also possible influences due to other differences in culture setup such as scaffold size, cell type and cell seeding number.

On the other hand, the inverse relationship between flow rate and the induced cell proliferation observed in the experiment was in agreement with the simulation results from Figure 4-5. Both experimental and simulation work demonstrated that the effect of flow on cell proliferation diminished when the flow rate exceeded 0.02 cm/s.

6.5.3.5 Limitations of the experiment

Fibroblasts impose contractile forces on ECM and scaffold structures in contact. Due to the contractile forces of the cells, the collagen scaffolds in the experiment shrank and became smaller after culture. The longer the culture, the greater the shrinkage. The size reduction of scaffolds led to an increasing gap between the scaffold and the culture chamber wall. Despite small, the gap could have resulted in flow channeling and the constructs were no longer evenly perfused. To avoid this, scaffold of stiffer materials could be used in the future. Alternatively, collagen scaffold can be glued to culture chamber wall at the beginning of culture to prevent scaffold deformation. For both options, the change of scaffold properties presents different growth niches for cells and might stimulate cell responses different from that in the study.

The initial cell seeding number was different for the three flow rate studies in the study. While cell seeding number directly affects the cell population on scaffold outer surface, it is not clear how it affects local cell growth. Studies on the effect of cell number on 3D construct development are needed in the future.

The time duration for observation was limited to 1 week. Experiments for longer culture time could be conducted in the future to study cell behavior under long time culture condition.

Finally, mass exchange was not measured in the experiment. In the future studies, nutrient levels could be measured at different time points so as to understand the influence of perfusion on overall nutrient consumption of the culture.

6.5.4 CONCLUSION FOR EXPERIMENT 2

Experiment 2 has examined cell growth under perfusion culture with three different perfusion rates. Statically cultured constructs were used as controls. It was found that perfusion helped promote cell proliferation in a perfusion rate dependent manner. The lower the perfusion rate, the better the cells were stimulated for proliferation. Cell growth tended to cease at a later part of the culture, which was linked to increasing local cell density and uneven cell distribution in the construct. Among the three flow rates: 1 ml/min, 2 ml/min and 3 ml/min, cells had the best growth profile under 1 ml/min. At 3 ml/min, only slightly more cells were resulted in scaffolds compared to control. The experiment suggested that perfusion rate for 3D construct development should be designed within a suitable range in order to optimize its stimulation effect for cell proliferation.

6.6 SUMMARY

This chapter has presented two *in vitro* experiments utilizing perfusion culture for 3D construct development. Experiment 1 examined the cell retention under perfusion. It was found that both perfusion rates and direction could influence the cell retention in the construct. Experiment 2 compared cell growth under different perfusion rates. It was found that perfusion culture stimulated cell proliferation and

alignment. Smaller perfusion rates resulted in better culture results. The experimental findings in this chapter assist tissue engineers to better understand the cell detachment at the starting stage of perfusion culture and cell growth under certain ranges of perfusion rates (1 ml/min to 3ml/min) for 3D construct development.

7 CONCLUSION AND FUTURE WORK

7.1 CONCLUSION

Tissue engineering provides a promising approach for treating tissue loss and damage. However, 3D tissue engineering faces the challenge of inadequate mass exchange in the inner construct region, which leads to uneven construct development and limits the construct size. To solve the problem, dynamic culture which helps deliver flow and nutrients into the construct should be utilized.

This research has explored perfusion culture for 3D construct engineering with both mathematical and experimental analysis. There are several key contributions of the work as follows:

- 1) In perfusion culture, flow field modulates the biochemical and biophysical cues for cell growth. This work has numerically studied the flow field related to modular designs of the perfusion system. The obtained knowledge helps tissue engineers to determine suitable perfusion system features for achieving desired flow fields in 3D construct culture.
- 2) An original mathematical model has been proposed to describe the cell growth kinetics under perfusion culture. It helps quantify the flow effect on cell growth in 3D constructs with consideration of both flow shear and nutrient effects.
- 3) A novel perfusion bioreactor has been designed and constructed for 3D construct culture in tissue engineering based on the modeling results.
- 4) Experiments have been conducted to investigate the 3D construct development under perfusion culture *in vitro*. Two important aspects, cell retention and growth, were examined to evaluate the construct growth

outcome under different perfusion durations, directions and rates. The experimental findings assist tissue engineers to better understand the cell behavior in 3D constructs under perfusion culture.

7.1.1 FLOW FIELD IN PERFUSION SYSTEM

Flow field in perfusion culture is largely determined by the bioreactor system design. This work has mathematically analyzed its dependence on some modular designs of the perfusion bioreactor system. Based on the modeling, the conical inlet design facilitated gradual flow divergence at the inlet region. It induced a more homogeneous flow field in the culture space compared to the cylindrical inlet design. With the cylindrical inlet design, a flow zone between the inlet/outlet and the scaffold was proven necessary in order to obtain even flow culture for the scaffold. To prevent flow channeling, the sizes of the scaffold and the perfusion chamber should be matched with each other. Internal channels of the scaffold could help improve the culture condition in the channel and adjacent scaffold regions, but did not provide a complete solution for solving the flow delivery problem of 3D constructs. Their influences to the flow field in the construct changes with channel opening design, orientation and size.

7.1.2 CELL GROWTH KINETICS UNDER PERFUSION CULTURE

Studying cell behavior in 3D constructs at the cellular level experimentally is extremely difficult due to the limitations of current sensing technologies. Mathematical modeling provides a very useful and convenient alternative. The work has proposed an original mathematical model that considered both the biophysical and biochemical effects of perfusion culture for 3D construct development. While the other existing models mainly focused on the biochemical effects, the proposed model provides a more accurate description for the cell growth kinetics under flow

stimulation. The modeling results revealed that dynamic culture could improve the culture results greatly even at flow rates as low as 0.002cm/s. Higher flow rates supported larger cell number growth, more even cell distribution and better mass exchange. Calculation with consideration of shear stress derived higher cell numbers in the construct compared to that without consideration for flow shear.

7.1.3 DEVELOPMENT OF A NOVEL PERFUSION BIOREACTOR SYSTEM

This work has developed a novel perfusion bioreactor system for 3D construct development. The developed bioreactor was biocompatible and allowed direct visual inspection during culture process. A special flow buffer zone was designed between the flow inlet/outlet and the scaffold to help flow diverge and reach an even distribution at the scaffold surface. Easy loading and harvest of scaffold were enabled by a simple assembly design of the culture vessel. The flow mechanics in the bioreactor confirmed the suitability of the bioreactor for 3D construct culture.

7.1.4 IN VITRO PERFUSION CULTURE OF 3D CONSTRUCTS

Two individual experiments have been carried out with the developed perfusion bioreactor to investigate 3D construct development under perfusion culture. Cell retention in the construct was found to be affected by the flow rate and flow direction. While the flow rate effect was largely linked to flow shear stress, the flow direction effect was linked to the uneven cell distribution in the construct. Cell proliferation in the construct was found to be dependent on flow rate, perfusion duration and the local cell density. In general, the stimulation effects of flow on cell proliferation were inversely related to the flow rate. Higher flow rates induced relatively slower cell number increase in the perfused construct compared to lower

flow rates. SEM observations revealed cell alignment on the perfused construct. The greater the perfusion rate, the faster the cells aligned.

The findings in this research have provided great insights on how perfusion culture should be designed to optimize the culture outcome and how cell growth in 3D constructs can be affected by perfusion culture.

7.2 FUTURE WORK

There is great potential for utilizing perfusion culture for 3D construct development in tissue engineering. Some future work of the research can be carried out as follows:

- 1) In 3D constructs, cells usually distribute unevenly due to uneven cell seeding. CFD models should be generated in the future to take the uneven cell distribution into consideration so as to provide a more realistic modeling for the culture.
- 2) More efficient seeding methods need to be developed for more even cell seeding in the 3D constructs. Perfusion bioreactor can be utilized for the purpose [193].
- 3) In Experiment 2 of the work (Section 6.5), different cell seeding densities were used for different perfusion groups. It is not clear whether such setup was linked to the observed difference in culture results. Further studies should be carried out to investigate the effect of cell number on cell proliferation and construct development under perfusion.
- 4) In the experimental study (Chapter 6) of the work, the shear and nutrient effects on cell growth were coupled. To better understand the respective effects of the two factors, experiments should be designed to decouple the

two factors. This could be done by modifying the medium viscosity while keeping the flow rate constant, which corresponds to changed flow shear but consistent nutrient supply.

7.3 LIST OF PUBLICATION

The publications generated from the research work are as follows.

7.3.1 JOURNAL PAPER

7.3.1.1 Published

Liu D, C.-K. Chua, et al. (2010). "Fibroblast response to interstitial flow: A state-of-the-art review". *Biotechnology and Bioengineering* 107(1): 1-10.

7.3.1.2 Submitted

1. Liu D, Chua CK and Leong KF. "Impact of perfusion on cell population for three-dimensional bioconstruct development". Submitted to *Journal of Tissue Engineering and Regenerative Medicine*

7.3.1.3 Under revision

1. Liu D, Chua CK and Leong KF. "A mathematical model for flow shear sensitive tissue engineering". Under revision for *Biomechanics and Modeling in Mechanobiology*

7.3.2 CONFERENCE PAPER

1. Chua CK, Liu D and Leong KF, "A minibioreactor for developing perfused blood vessels in cardiomyocyte aggregates". 4th International Conference on Advanced Research in Virtual and Rapid Prototyping, Leiria, Portugal, 6 to 10 Oct 2009.

2. Liu D, Chua CK and Leong KF. "Fluid flow design for tissue engineering: the aspects of shear stress and nutrient supply". International Conference on Cellular & Molecular Bioengineering (ICCMB2). Singapore, 2-4 August 2010.
3. Liu D, Chua CK and Leong KF. "Impact of dynamic perfusion on cell population in 3D construct development". 1st International Symposium on Bioengineering, Nanyang Technological University. Singapore, 19-20 December 2010.

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APPENDIX A

Commercial tissue engineering products at various stages of development [6]

Tissue	Product	Regulatory status	Description	Material						Cells	Use	Form
				Synthetic	Resorbable	Animal derived	Plant or bacteria derived	Human derived	Growth factor			
Skin	TransCyte, Advanced Biohealing	1997	Nylon mesh coated with porcine collagen, containing non-viable human fibroblasts, with upper layer of silicon	✓	✓	✓				✓	Burns	Sheet
	Apligraf, Organogenesis	1998	Lower layer of human fibroblasts and bovine collagen, upper layer of keratinocytes		✓	✓				✓	Leg ulcers	Sheet
	Dermagraft, Advanced BioHealing	2001	Cryopreserved human fibroblasts on a polyglactin 910 (2-hydroxy-propanoic acid polymer with polymerized hydroxyacetic acid) mesh	✓	✓					✓	Diabetic foot ulcers	Sheet
	ICX-SKN, Intercytex	Phase II	Allogenic fibroblasts and human collagen with additional layer of keratinocytes		✓			✓		✓	Burns and acute wounds	Sheet
	Integra Dermal Regeneration Template, Integra Lifesciences	1996	Porous bovine collagen crosslinked with chondroitin-6-sulphate with upper layer of silicon	✓	✓	✓					Burns	Sheet
	Integra Flowable Wound Matrix, Integra Lifesciences	2007	Granulated bovine collagen crosslinked with chondroitin-6-sulphate		✓	✓					Ulcers	Gel
	Oasis Wound Matrix, Healthpoint	2006 ^a	Decellularized porcine small intestinal submucosa		✓	✓					Burns, ulcers, other wounds	Sheet
	PriMatrix, TEI Biosciences	2008	Decellularized fetal bovine skin		✓	✓					Wounds	Sheet
	Xelma, Molnlycke	2005 EU	ECM protein (amelogenins) in propylene glycol alginate carrier		✓	✓	✓				Leg ulcers	Gel
Bone	INFUSE Bone Graft, Medtronic	2002	Bovine type I collagen sponges soaked in rhBMP-2 in LT-CAGE Lumbar Tapered Fusion Device	✓	✓	✓				✓	Spinal fusion	Solid
	OP-1, Stryker	2001	Bovine type I collagen with rhBMP-7		✓	✓				✓	Bone injury	Paste
	PuraMatrix, 3DM	Preclinic	Synthetic 16-amino-acid peptide, forming nanofibres	✓	✓						Dental bone defects	Gel
	Vitoss Scaffold FOAM, Orthovita	2004	Porous foam comprising β -TCP and bovine type I collagen	✓	✓	✓					Bone injury	Foam
	Bioset IC, Pioneer surgical	2008	Human demineralized bone matrix with bovine bone chips in type I collagen carrier		✓	✓		✓			Bone injury	Paste
	FortrOss, Pioneer Surgical	2008	Nanocrystalline hydroxyapatite and E-matrix (porcine collagen co-polymerized with dextran)	✓	✓	✓	✓				Bone injury	Paste
	Regenafil, Regeneration Technologies/Exatech	2005	Human mineralized bone matrix in porcine gelatin carrier		✓	✓		✓			Bone injury	Paste
	GEM 21S, BioMimetic Therapeutics	2005	β -TCP particles and recombinant human platelet-derived growth factor-BB (PDGF-BB)	✓	✓				✓		Dental bone/gum defects	Paste

Commercial tissue engineering products at various stages of development [6] (continued)

Tissue	Product	Regulatory status	Description	Material						Cells	Use	Form	
				Synthetic	Resorbable	Animal derived	Plant or bacteria derived	Human derived	Growth factor	Allogenic	Autologous		
Cartilage	Synvisc, Genzyme	1997	Hyaluronic acid (Hylan GF-20 and Hylan B) from chicken combs	✓	✓							Synovial fluid replacement	Gel
	CaReS, Arthro Kinetics	2007 Germany	Rat-tail type I collagen matrix seeded with chondrocytes	✓	✓					✓		Articular cartilage injury	3D disc
	Bioseed-C, Biotissue Technologies	Clinic 2001 ^b	Polyglycolic/polylactic acid and polydioxane scaffold containing chondrocytes	✓	✓					✓		Articular cartilage injury	3D disc
	Menaflax, Regenbiologics	2008	Bovine type I collagen with hyaluronic acid and glycosaminglycans, hydrated	✓	✓							Meniscus cartilage injury	Mesh
	Hyalograft C autograft, Fidia advanced biopolymers	2008 EU ^c	HYAFF (esterified derivative of hyaluronic acid) scaffold with autologous chondrocytes	✓			✓			✓		Articular cartilage injury	3D disc
	MACI, Genzyme	Unreg EU ^d	Type I collagen membrane seeded with chondrocytes	✓	✓					✓		Articular cartilage injury	Sheet
T/L	GraftJacket, Wright Medical Technology	Unreg ^e	Decellularized human skin	✓				✓				Tendon and ligament repair	Sheet
	X-Repair, Synthasome	FDA review ^f	Poly-L-lactic acid woven mesh	✓	✓							Tendon and ligament repair	Sheet
BV	VascuGel, Pervasis	Phase II	Gelfoam porcine gelatin foam sponges seeded with endothelial cells	✓	✓					✓		Vessel reconstruction	Tubular
	Vascu-guard, Synovis	1994	Decellularized bovine pericardium	✓	✓							Vessel reconstruction	Sheet
HV	Cryovalve SG pulmonary human heart valve, Cryolife	2008	Decellularized human heart valves	✓				✓				Heart valve replacement	Valve
Retina	NT-501, Neurotech	Phase II/III	Genetically modified cells secreting ciliary neurotrophic factor in hollow permeable polyethersulphone fibres	✓						✓		Retinitis Pigmentosa	Tubular
Nerve	NeuraGen, Integra	2001	Semipermeable bovine type I collagen nerve conduits	✓	✓							Nerve injury	Tubular
Pancreas	Islet Sheet, Cerco Medical	Preclinic	250-µm-thick alginate sheet with encapsulated pancreatic islets (pig or human)				✓			✓		Diabetes mellitus	Sheet
	Amcyte/ReNeuron	Phase II	Alginate/poly-L-lysine encapsulated human pancreatic islets	✓			✓			✓		Diabetes mellitus	Bead
	Novocell	Phase I/II	Alginate/polyethylene glycol encapsulated human pancreatic islets	✓			✓			✓		Diabetes mellitus	Bead
Bladder	Neo-bladder, Tengion	Phase II	Poly(lactic-co-glycolic acid) seeded with urothelial and smooth muscle cells	✓	✓					✓		Spina bifida, bladder dysfunction	Sheet
Dura	DurADAPT, Pegasus Biologicals	2005	Decellularized, crosslinked equine pericardium	✓	✓							Reconstruction of dura mater	Sheet
Various	Extracel, Glycosan Biosystems	Preclinic	Crosslinkable bacterial hyaluronic acid, bovine and porcine gelatin, porcine heparin and PEG derivatives	✓	✓	✓	✓		g	g	g	Examples: cartilage, bone, vocal fold TE	Gel, sheet, tubular

Tissue abbreviations: T/L, tendon and ligament; BV, blood vessel; HV, heart valve. Regulatory status: dates indicate year approved by regulatory body (FDA unless stated as European Union (EU) or Germany). Preclinic indicates preclinical development, phase I and phase II indicate clinical trial stage. Date of regulatory approval does not necessarily coincide with market release. ^aReclassified in 2006 from an earlier product. ^bFirst clinical trials were in 2001. ^cCommercial sale began in 2000. ^dUnregulated in EU, not available in the United States. ^eUnregulated product. ^fDecision expected 2009. ^gAddition of growth factors and cells is dependent on application.

APPENDIX B

Statistical analysis for experimental data from Experiment 1 of Chapter 6

	perfusion flow rate					
Group	0ml/min	2.3ml/min	3.6ml/min	4.9ml/min	6.1ml/min	
perfused from top surface	0.3386	0.3654	0.2900	0.3126	0.3090	
	0.3556	0.2917	0.2832	0.2835	0.2924	
	0.2953	0.3047	0.2939	0.2595	0.2959	
	0.3416	0.3117	0.2675	0.2885	0.3386	
perfused from bottom surface	0.3386	0.3028	0.3086	0.3045	0.2932	
	0.3556	0.3118	0.3068	0.2803	0.2957	
	0.2953	0.2756	0.2749	0.2537	0.2934	
	0.3416	0.3143	0.2602	0.2523	0.3156	
Anova: Two-Factor With Replication		alpha=0.05				
SUMMARY	0ml/min	2.3ml/min	3.6ml/min	4.9ml/min	6.1ml/min	Total
<i>perfused from top surface</i>						
Count	4.0000	4.0000	4.0000	4.0000	4.0000	20.0000
Sum	1.3310	1.2734	1.1345	1.1440	1.2359	6.1189
Average	0.3328	0.3184	0.2836	0.2860	0.3090	0.3059
Variance	0.0007	0.0011	0.0001	0.0005	0.0004	0.0008
<i>perfused from bottom surface</i>						
Count	4.0000	4.0000	4.0000	4.0000	4.0000	20.0000
Sum	1.3310	1.2045	1.1504	1.0908	1.1979	5.9746
Average	0.3328	0.3011	0.2876	0.2727	0.2995	0.2987
Variance	0.0007	0.0003	0.0006	0.0006	0.0001	0.0008
<i>Total</i>						
Count	8.0000	8.0000	8.0000	8.0000	8.0000	
Sum	2.6621	2.4779	2.2850	2.2348	2.4338	
Average	0.3328	0.3097	0.2856	0.2794	0.3042	
Variance	0.0006	0.0007	0.0003	0.0005	0.0003	

Statistical analysis for experimental data from Experiment 1 of Chapter 6

(continued)

ANOVA						
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Sample	0.0005	1.0000	0.0005	1.0246	0.3195	4.1709
Columns	0.0143	4.0000	0.0036	7.0519	0.0004	2.6896
Interaction	0.0006	4.0000	0.0002	0.3148	0.8658	2.6896
Within	0.0152	30.0000	0.0005			
Total	0.0307	39.0000				

Fisher's Least Significant Difference (LSD) Test					
modified two-tail t test					
alpha=0.05		$t_{0.05(30)}=2.042$		delta	power of the statistical model
H0: u1=u2	t=	1.4439		1.8393	0.4500
H0: u1=u3	t=	2.9574	reject	4.4658	>0.85
H0: u1=u4	t=	3.3506	reject	4.5543	>0.85
H0: u1=u5	t=	1.7902		2.7737	0.7900
u1,u2,u3 ,u4 and u5 are means of samples under 0 ml/min, 2.3ml/min, 3.6ml/min, and 6.1ml/min perfusion respectively					