Development of collagen scaffold with internal channels via indirect rapid prototyping

Yeong, Wai Yee

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Development of Collagen Scaffold with Internal Channels via Indirect Rapid Prototyping

Yeong Wai Yee

School of Mechanical and Aerospace Engineering

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ABSTRACT

In this project, a commercial 3-D inkjet printer, a representative Rapid Prototyping (RP) system, was employed to produce collagen scaffold using an indirect, sacrificial mould method. This method offers a great degree of design freedom compared to conventional as well as direct RP-fabrication method. To better understand the inkjet printing process, a physical model was established to relate the operating parameters to the dimensions of the part printed. The ability to control the parameters is important in order to achieve the predetermined morphology and resolution of the mould in this indirect fabrication technique.

One major challenge in scaffold-based tissue engineering has been the limitation of cell migration and tissue ingrowth within the scaffolds. In this research, networks of channels were incorporated into the matrix as part of the architecture to overcome the limitation of nutrient diffusion. The shape, dimension, distribution, configuration as well as the orientation of the channels can be easily designed and controlled. The calculations of the channels lengths were performed based on the estimated flow shear in the channels and the oxygen mass balance required in the scaffold. An analytical model is presented to illustrate the enhancement of cell proliferation in scaffold with the internal channels.

In vitro cell culture experiments with Human Primary Osteogenic Sarcoma (SaOS-2) cells were performed on control collagen scaffolds without internal channels and RP-fabricated collagen scaffolds with internal channels. Cell Proliferation Assay studies showed significantly more cells were attached within the scaffolds with internal channels and these cells were distributed more homogenously. Scanning electron microscopy analysis and histological analysis confirmed that cells were attached in the interior region of the RP scaffolds. Higher cell penetration depth was also observed in the scaffolds through this improved technique.

A customized direct perfusion bioreactor for the cultivation of cells on compliant scaffold was developed successfully. Significantly higher cell density was recorded in direct perfusion cultured scaffolds compared to the static cultured ones. The

combination of a RP-fabricated collagen scaffold and the direct perfusion bioreactor was able to maximize the proliferation of cells in scaffold and enhanced homogeneous distribution of cells.

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LIST OF SYMBOLS

A	Cross section of a line
A_{sc}	Area of scaffold
b_{sc}	Width of scaffold control volume
c	Acoustic velocity
C_{in}	Concentration of oxygen at the inlet
C_{out}	Concentration of oxygen at the outlet
C_{O_2}	Oxygen concentration
D	Distance between the droplets
d_{cell}	Diameter of cell
d_{ch}	Diameter of flow channel
d_m	Maximum spread diameter of droplet
d_o	Initial diameter of droplet
D_{O_2}	Diffusivity of oxygen
d_p	Peak-to-peak value
d_{sc}	Diameter of scaffold
E	Young's modulus
f	Frequency of droplet ejection
h	Thickness of scaffold
h_{drop}	Droplet height
h_{line}	Height of line
h_{sc}	Height of scaffold control volume
I	Edge area
J	Diffusion flux
k	Proportional constant
KE	Kinetic energy
L	Length of scaffold element
L_{sc}	Characteristic length of scaffold
l	Length of the tube
m	Constant

N_{cell}	Number of cells
N_{drop}	Number of droplets
$\hat{N}$	Cells per volume
P	Generated stress
Q	Flow rate
R_a	Surface roughness
Re	Reynolds number
R_{O_2}	Oxygen consumption rate
R_{sphere}	Radius of the spherical cap
S	Thiele ratio
s	Distance traveled by the printhead
SE	Surface energy
t	Time
T	Residence time
U	Scanning speed of the printhead
V	Velocity
V_{ef}	Applied electric field
V_m	Velocity of internal fluid flow
Vol_{cell}	Volume of cell
Vol_{cap}	Volume of the spherical cap
Vol_{drop}	Volume of the droplet
Vol_{net}	Net volume available for cells occupation
W	Work done
w	Width of line
We	Weber number
x	Distance
ρ	Material density
μ	Viscosity
σ	Surface tension
τ	Fluid shear stress
ε	Induced strain
α	Piezoelectric coefficient

θ	Contact angle
ξ	Spread ratio
ϕ	Void fraction
ϕ_{liq}	Porosity measured by liquid displacement method
ϕ_{gas}	Porosity measured by gas displacement method
ψ	Ratio of concentration

CHAPTER 1

INTRODUCTION

1.1 Background

Tissue engineering has gained attention in the past decades owing to the apparently vast commercial potential offered by the field. The term Tissue Engineering (TE) is defined as the “application of the principles and methods of engineering and life sciences toward fundamental understanding of structure-function relationship in normal and pathological mammalian tissues and the development of biological substitutes for the repair or regeneration of tissue or organ function” [1].

It is an interdisciplinary field that integrates discoveries from biochemistry, cell and molecular biology, genetics, material science and biomedical engineering to produce patient-specific biological substitutes that can circumvent the limitations of existing clinical therapies and treatments for damaged tissue or organs. These limitations include shortage of donor organs, issue of chronic rejection and cell morbidity [1].

The main regenerative tissue engineering approaches include injection of cells alone, development of encapsulated systems and transplantation of cells in scaffold [2]. The last approach appears to be the dominant method in the research of tissue engineering because it can be realized easily in the short term and permits experimental manipulation at three levels to achieve optimal construct: the cells, the polymer scaffolds and the construction methods [2].

Scaffolds used in regeneration of tissues or organs serve the purpose of providing cells with a suitable environment for cells seeding and proliferation. Tissues are characterized by a well-defined three-dimensional (3-D) arrangement of cells. Spatial control of scaffold elements may be used to encourage the organization of cells to conform to those resembling the natural tissue that it is regenerating. The populated TE scaffolds are eventually implanted into the human body for the treatment and recovery of the diseased tissues or organs.

Scaffolds can be produced in a variety of ways. In general, the methods can be categorized into conventional techniques or advanced processing methods. Traditional methods for controlling the scaffold architecture include solvent casting particulate leaching [3], gas foaming [4], fibre meshes/fibre bonding [5], phase separation [6], melt moulding [7], emulsion freeze drying [8], solution casting and freeze drying [9]. However, there are inherent limitations in these processing methods [10], particularly that control over scaffold architecture using these conventional techniques is highly process dependent instead of design driven. In view of such limitations, advanced methods such as rapid prototyping (RP) are seen to be viable alternatives to achieve extensive and detailed control over scaffold architecture [10, 11].

RP produces physical objects in a layer-by-layer manufacturing process from a computer-generated model. The application of RP to tissue engineering could bring significant breakthroughs in the field due to the possibility of controlling the macro- and micro-architecture of the scaffold so as to favour cell growth [11].

Another pivotal element in scaffold-based tissue engineering is the culturing strategy adopted to ensure uniform cellularity of the scaffold and to sustain high cell density in the scaffold. Static culturing technique is found to be inefficient in the cultivation of a 3-D scaffold due to the nutrient diffusion limits. As a result, bioreactor systems are being developed as a potential device to create optimal biomechanical and biochemical conditions for the cultivation of cell-seeded 3-D scaffolds.

Bioreactors are generally defined as devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions (e.g. pH, temperature, pressure, nutrient supply and waste removal) [12]. Some representative bioreactors for tissue engineering applications include spinner-flasks [13], rotating-wall vessels [14], hollow-fibre bioreactors [15], direct perfusion bioreactors [16-18] and bioreactors that apply controlled mechanical forces such as dynamic compression and cyclic strains [19]. Hydrodynamic conditions in bioreactors such as fluid flow-induced shear stress and pressure were found to have enhanced cellular and matrix distributions [20, 21].

1.2 Motivation

The scaffold-based approach in tissue engineering often has to deal with the issue of successful cellurization of the scaffold. When cells are first transplanted on a 3-D porous scaffold, the area within the scaffold is avascular and the transplanted cells are dependent on the diffusion of nutrients and waste for survival [22]. This diffusion is adequate only if the tissue is small (< 1 mm thick) or the cells' metabolic needs are low. But in the case of engineering an artificial organ, such as the liver, most cells will die soon after transplantation because of mass transport limitations [22].

One solution to overcome this limitation is to stimulate the rapid development of a neo-vascular network within three-dimensional tissue engineering matrices, incorporating flow channels into the matrix as part of the architecture. Such matrix with intricate internal morphology can be achieved with the application of RP techniques.

Currently available RP technologies operate with a limited number of materials such as synthetic polymers and certain ceramics as the standard working material. If non-standard materials such as thermally sensitive natural biomaterials are to be utilized, an indirect RP method using sacrificial mould is often required to transfer the morphology of the fabricated part into the desired target material. This is because some RP systems use elevated temperature to fuse the material with the purpose of producing a continuous 3-D part.

The advantages of natural biomaterials include: firstly, their properties closely simulate that of the natural extracellular matrix, secondly, they are non-toxic, and finally, they have a more native surface relative to the synthetic polymers that may positively support cell adhesion and function [23].

Based on the criteria of utilizing natural biomaterial and achievable resolution, a 3-D inkjet printer (Solidcape T612) was selected as the primary vehicle in this work. This RP process provides a significant advantage in that the fabricated part, which serves as a sacrificial mould, can be removed by dissolution. The original properties of the

biomaterial are well preserved as no heating process is imposed on the scaffold material.

As in any design problem, the development of scaffold using indirect RP is an iterative process. Three main issues in the application of indirect RP techniques to fabricate TE scaffold are thorough understanding of the chosen RP technique, the design of the scaffold and the application of the system to fabricate scaffold. Important factors are the capability of the RP process, the selection of material and the processing conditions required for the realization of scaffold (see Figure 1.1).

Figure 1.1: Interrelationships of RP and TE scaffold.

Each segment requires the knowledge input of the related segments in order to achieve fruitful results. Hence, one primary motivation for this work is to understand and model these relationships so as to produce patient-specific scaffolds as well as overcoming the challenges of optimal vascularization.

The second stage of this project focuses on the development of a dynamic culturing strategy to maximize the potential of the scaffolds fabricated.

Conventional static cultures of 3-D cell-scaffold constructs in culturing dishes are found to be inadequate in several aspects. For example, polymeric scaffolds cultivated with stromal osteoblasts in static cultures had inhomogeneous cell and matrix distribution throughout the scaffolds [24]. A thin layer of cells is observed at the exterior of the scaffolds while leaving the pores at the center of the scaffolds empty.

To overcome this problem, a direct perfusion bioreactor will be developed in-house. Bioreactors that utilize the direct perfusion chambers have the ability to enhance diffusion and apply orientated flow-induced shear stress on the construct at the same time.

1.3 Objectives

The primary goal of the research aims at developing the capability to fabricate collagen scaffolds with controllable internal architecture using advanced manufacturing process. It is also another goal of this research to develop the enabling technology for successful cultivation of these scaffolds.

The detail objectives of this project are:

- (i) To understand and model the 3-D inkjet printing process so as to gain the operating insight of and determine the optimal conditions for the process.
- (ii) To explore the production of scaffolds by an indirect method and hence design a sacrificial mould with inter-connected internal channels as a negative of the scaffold.
- (iii) To produce a highly porous structure for cell immobilization and a 3-D channel network of channels acting as flow channels for homogeneous perfusion of culture medium over the scaffold.
- (iv) To design and develop a dynamic culturing technique to maximize the *in vitro* performance of the collagen scaffolds.

1.4 Scope

In this project, 3 important areas of research will be involved namely the design of scaffold, fabrication of scaffold and development of bioreactor. The detail scope of the project includes the following:

(i) Study the operating process of droplet-based manufacturing technique to establish a mathematical model for a better understanding and identify the important parameters.

(ii) Design and fabricate suitable sacrificial moulds to shape the scaffold.

(iii) Develop a suitable and consistent processing technique to transfer the pre-determined morphology to the scaffold.

(iv) Evaluate the microstructure of the scaffolds fabricated using a selected biomaterial, which, in this project, is collagen.

(v) Design and develop a direct perfusion bioreactor for dynamic cultivation of scaffolds.

(vi) Assess the performance of the collagen scaffolds under static and dynamic culturing conditions in terms of cell proliferation and distribution.

(vii) Summarize the process and protocol as a novel, viable and fully self-contained methodology to produce functional tissue engineering scaffolds.

1.5 Organization of Thesis

The thesis is organized as follows:

Chapter 1 presents a brief introduction to tissue engineering, the background and motivation for this project. Consequently, the objectives and scope of the project are established.

Chapter 2 consists of literature reviews that cover four main aspects of this research, namely, tissue engineering, TE scaffold, rapid prototyping and bioreactor. Biomaterials that have been used in TE are also described. Critical issues and challenges in the scaffolding technology are identified and discussed. This chapter also presents a comprehensive review on the application of RP in TE scaffold. Different types of bioreactor are described as well as the challenges faced in the development of a bioreactor in tissue engineering.

Chapter 3 discusses the theory underlying the 3-D inkjet printing technology with some experimental work. The modelling of the 3-D inkjet printing process is presented. The analysis is useful in the prediction of the printing performance, material selection, optimum configuration of parameters and investigation of the effect of specific process variables.

Design calculations of the sacrificial mould are presented in Chapter 4. An analytical model is also described at the end of this chapter to illustrate the enhancement of cell proliferation in scaffold with the internal channels.

The methodology undertaken in the research is presented in Chapter 5. The processing steps of the scaffold are presented and a comparative study on two different drying techniques is also presented. Various types of analysis on the characterization of the scaffold are also outlined. The characterizations were performed on (a) the mould, (b) the scaffold, and (c) the cell-seeded scaffold. The cell culture protocol and the characterization techniques for *in vitro* evaluation of the collagen scaffolds are presented. Two different culturing techniques namely the static and the direct perfusion culture are described. Analyses include routine mycoplasma

detection, MTT-based cell proliferation assay, scanning electron micrograph analysis and histological analysis for the evaluation of cell proliferation and distribution in scaffolds.

Chapter 6 details the design and development of a direct perfusion bioreactor used in this project. The design calculation and the development of modular components are documented. Bioreactors that utilize the direct perfusion chambers have the ability to enhance diffusion and apply orientated flow-induced shear stress on the construct at the same time.

In Chapter 7, the results of the various experiments are presented. Protocol of the novel indirect fabrication technique is reported. Subsequent discussion focuses on the morphology of the control collagen scaffold fabricated by conventional technique, characterization of the 3-D inkjet printed mould, followed by a comprehensive analysis of the RP fabricated collagen scaffold with internal channels. The *in vitro* application of the bioreactor with RP scaffolds is discussed. The hydrodynamic of the bioreactor is modeled analytically to estimate the fluid shear stress in the pores of the scaffold.

Conclusion and future works are described in Chapter 8. Finally, a list of publications resulting from this work is presented.

CHAPTER 2

LITERATURE REVIEW

The literature reviews in this chapter focus on four main aspects of research, namely, tissue engineering, tissue engineering scaffold, application of rapid prototyping in tissue engineering and the development of bioreactor for cell-scaffold cultivation.

The requirements of scaffold and the conventional fabrication methods of tissue engineering scaffold are discussed in Section 2.1 and 2.2. Biomaterials that have been used in TE are also described in Section 2.3. The requirements of scaffold are classified into 7 critical issues, namely: (1) Choice of material, (2) Effect of porosity (3) Effect of pore size (4) Effect of pore interconnectivity (5) Effect of degradation rate (6) Bioactivity of scaffold and (7) Consistency of processing method. The detailed discussions are presented in Section 2.4.

Section 2.5 presents a comprehensive review on the advanced methods of scaffold fabrication using rapid prototyping (RP). The RP technologies are categorized following the mode of assembly, namely melt/dissolution deposition technique, particle bonding technique and indirect fabrication method. Potential and challenges of scaffold-based technology are discussed from the perspective of RP technology in Section 2.6.

Bioreactors are an important enabling technology in scaffold-based tissue engineering for successful cultivation of cells on scaffolds. Section 2.7 discusses the different types of bioreactor developed in tissue engineering. The challenges of bioreactor are described in Section 2.8.

2.1 Tissue Engineering

Scaffold-based tissue engineering allows one to precisely define the microenvironment (e.g., cell types, matrices, growth factors) in which tissues are developing. There are four important considerations involved in the approach of scaffold-based tissue engineering, namely the cell source, the scaffold itself, cultivation of cell-scaffold constructs and implantation techniques (see Figure 2.1).

Figure 2.1: Scaffold-based tissue engineering [25].

In the attempt to produce functional tissues, specific cell populations are harvested from the appropriate tissue and seeded on a biodegradable polymer scaffold. Growth factor is incorporated to enhance the proliferation of cells on the scaffolds. The cell/polymer constructs may undergo a period of dynamic tissue culture in a bioreactor prior to implantation.

Extensive research has been carried out in the field of tissue engineering in the quest for suitable tissue engineered organs or tissues such as skin [26], liver [27], cartilage

[28] and bones [29]. However, different kinds of tissue modes would require a complete set of design specifications for the scaffold.

2.2 Tissue Engineering Scaffold

Biological observations show that all tissues constantly remodel themselves and dissociated mature cells can reorganize themselves into their native structure when placed in an appropriate cell culture [25]. However, cells lack the ability to grow in their favoured 3-D orientations that define the anatomical shape of the tissue. Therefore, cell suspension needs a template to guide restructuring to achieve reorganization.

The scaffold tries to mimic the function of the natural extracellular matrix (ECM) and hence is sometimes referred as a synthetic ECM. The primary roles of a scaffold are [30]:

- to serve as an adhesion substrate for cell, facilitating the localization and delivery of cells when it is implanted,
- to provide temporary mechanical support to the new grown tissue by defining and maintaining a 3-D space and
- to guide the development of new tissues with appropriate functions.

2.2.1 Requirements of scaffold

Successful scaffolds should possess the following desirable characteristics [10]:

1. A suitable macrostructure to promote cell proliferation and cell-specific matrix production.
2. An open-pore geometry with a highly porous surface and microstructure that allows cell-ingrowth.
3. Optimal pore size employed to encourage tissue regeneration and to avoid pore occlusion.
4. Suitable surface morphology and physiochemistry properties in order to encourage intracellular signalling and recruitment of cell.
5. Made from non-toxic material with a predictable rate of degradation and no cytotoxic residues.

2.2.2 Fabrication method

Several different processing techniques have been developed for the design and fabrication of 3-D scaffolds suitable for TE implants. Conventional methods for controlling the scaffold architecture include solvent casting particulate leaching [3], gas foaming [4], fibre meshes/fibre bonding [5], phase separation [6], melt moulding [7], emulsion freeze drying [8], solution casting and freeze drying [9]. However, there are inherent limitations in these processing methods as summarized in a review by Leong *et al* [10]. A brief discussion on some of the common methods is presented below.

(a) Solvent casting-particulate leaching

This method involves dissolution of polymer in solvent and then pouring the polymer solution into a porogen-containing mould. Porous structure is obtained after allowing the solvent to evaporate and leaching out the porogens. Interconnectivity between pore is low and difficult to control. Pore walls often have uncontrollable morphologies and only a relatively thin scaffold (<1cm, in wafer or thin film form) can be produced due to the issue of entrapped porogen particle in a thick section.

(b) Gas foaming

Polymer is compressed into a solid mixture (pellet) of a discontinuous polymer disk, which was then exposed to high pressure CO₂ gas to saturate the polymer. Pressure is then released rapidly, creating thermodynamic instability that causes the polymer to foam. The resultant foam structure has a largely non-porous outer skin layer, which is undesirable. Further investigation reveals that a mixture of open and closed pores are formed within the center, leaving incomplete interconnectivity where only 10 to 30% interconnectivity is reported.

(c) Phase separation

Polymer is dissolved in a solvent and then cooled to induce liquid-liquid phase separation and quenched to create a two-phase solid. This method allows the incorporation of biomolecule into the scaffold owing to the low temperature process. However, the sublimation step may generate a dense skin on the open surface of the solution.

(d) Fibre meshes/fibre bonding

Fibres are bonded together in three-dimensions, to provide a large surface area for cell interaction and growth. Limitation lies in the diameter of the fibre used. The diameter of PGA fibre is around 15µm, which is far greater than the typical fibres diameter of natural extracellular matrix at the scale of 50-500nm. The structure also has low mechanical strength.

The advantages and disadvantages of each process are tabulated in Table 2.1[10].

Process	Advantages	Disadvantages
Solvent Casting & Particulate Leaching	Highly porous structures. Large range of pore sizes. Independent control of porosity and pore size. Crystallinity can be tailored.	Limited membrane thickness. Lack of mechanical strength. Problems with residual solvent. Residual porogens.
High-Pressure Processing	Organic solvent free. Allows incorporation of bioactive agents.	Nonporous external surface. Closed pore structure. Limited interconnected pores.
Fibre Bonding	Easy process. High porosity. High surface area to volume ratio.	High processing temperature for non-amorphous polymer. Limited range of polymers. Lack of mechanical strength. Problems with residual solvent. Lack of control over micro-architecture.
Phase Separation	Allows incorporation of bioactive agents. Highly porous structures.	Lack of control over micro-architecture Problems with residual solvent Limited range of pore sizes
Melt Moulding	Independent control of porosity and pore size. Macro shape control.	High processing temperature for non-amorphous polymer. Residual porogens.
Freeze Drying	Highly porous structures. High pore interconnectivity.	Limited to small pore sizes.

Table 2.1: Advantages and disadvantages of each fabrication process.

These processing methods offer little capability to precisely control pore size, pore geometry, pore interconnectivity, spatial distribution of pores and construction of internal channels within the scaffold. Consequently, researchers try to modify the conventional techniques to overcome these inherent process limitations.

2.2.3 Improved conventional method

Researchers try to modify the conventional techniques to circumvent those inherent process limitations described in Table 2.1. Kim and Mooney produced Poly(glycolic acid) (PGA) fibre bonded with poly (L-lactide acid) (PLLA) to enhance the mechanical strength of the unbonded PGA fibre meshes [31]. The degradation rate was also altered due to the bonding. Ho *et al* prepared scaffolds by using freeze-extraction and freeze-gelation methods as a variant of freeze-drying process, which is relatively more time and energy consuming [32]. Scaffolds fabricated are made of PLLA, poly(lactide-co-glycolic acid) (PLGA), chitosan and alginate.

William *et al* recognized the need for enhanced pore interconnectivity in a variety of tissue engineering applications [33]. They fused the porogen such as sodium chloride (NaCl) together prior to mixing into polymer matrix in solvent casting/particulate leaching and gas foaming/particulate leaching process. The result showed that holes were formed in pore walls, which indicated pores interconnectivity. Reignier *et al* reported a technique that involved the incorporation of NaCl salt particles in a blend of Poly (ϵ -caprolactone) (PCL) and polyethylene oxide (PEO) using melt processing techniques, followed by the selective extraction of the salt and PEO to leave a highly porous PCL scaffold [34]. Capes *et al* used sugar spheres to manufacture porous PLGA and resin scaffolds. The packing configuration of the sugar spheres was utilized to achieve layered and directional pores [35]. A similar concept is found in the work of Chen and Ma [36]. They created nano-fibrous PLLA scaffolds that incorporated interconnected spherical macropores. The macropores were left void by paraffin spheres which were thermally bonded before the casting of the polymer solution. In place of paraffin spheres, Gross and Rodríguez-Lorenzo used a sintered salt template to produce PLLA reinforced apatite scaffolds [37]. In a recent study, polyurethane scaffold was prepared by a combination of salt leaching and freeze-drying of dioxane to obtain porous scaffolds with a very high interconnectivity [38].

The abovementioned modifications show the intention of the researchers to control the internal architecture of the scaffold. The general idea is to create a network template before the casting of polymer. However, this approach offers limited degree of control in the aspect of three-dimensional internal architecture. The control over

scaffold architecture is highly process dependent instead of design driven. On the other hand, RP is relatively more flexible, offering a greater degree of control in the fabrication of intricate internal architecture. Detail discussion on these techniques is presented in Section 2.5.

2.3 Biomaterials

A biomaterial refers to “a material that is used in a medical device and intended to interact with biological systems” [39]. The role of biomaterials has evolved from being bioinert to bioactive inline with the development in tissue engineering applications [40]. Some common biomaterials such as polymers and ceramics are discussed. Polymer used as biomaterials can be naturally derived or synthetically produced.

2.3.1 Synthetic polymer

Some of the most commonly investigated synthetic polymers used as matrices for TE scaffold are described.

(a) Poly (glycolic acid), poly (lactic acid) and their copolymers:

PGA, PLA and their copolymers are the most widely used biodegradable polymers in medicine. The ester bonds in these polymers are hydrolytically labile, and these polymers degrade by nonenzymatic hydrolysis [41].

(b) Poly(ϵ -caprolactone) (PCL)

Poly(ϵ -caprolactone) (PCL) is a semi crystalline polymer with a melting point of around 60°C [42]. The degradation time of PCL is approximately two years.

2.3.2 Natural polymer

There are many different existing potential natural biodegradable-scaffold materials that may be used for TE scaffold applications. Some of the most commonly used materials that may be used as tissue scaffolds and their clinical application are discussed.

(a) Type I collagen

Collagen is the major protein component of mammalian connective tissue, accounting for 30% of all protein in the human body [43]. It is found in every major tissue that requires strength and flexibility. Clinical applications include the manufacture of sutures, haemostatic agents (powder, sponge, fleece), blood vessels by extrusion tube, tendons and ligaments, dermal regeneration for burn treatment and nerve guide for peripheral nerve regeneration [44].

(b) Glycosaminoglycans (GAGs)

Glycosaminoglycans are highly charged polycarboxylated, polysulfated polysaccharides molecules. The specific GAGs of physiological significance are hyaluronic acid, dermatan sulphate, chondroitin sulphate, heparin, heparan sulphate, and keratan sulphate [45].

(c) Chitosan

This biosynthetic polysaccharide can be slowly depolarised *in vivo* with lysozyme [46]. Chemical modification of chitosan produces material with a variety of physical and mechanical properties. Chitosan can be formed into membranes and matrices suitable for several TE applications such as nerve and skin regeneration [47, 48].

2.3.3 Ceramics

This category consists of inorganic, nonmetallic compounds that exhibit a variety of combinations of ionic and covalent bonding. They have been widely used in biomedical applications for load bearing implants such as total hip replacements and the dental industry. Most members of this group are characterized by a high Young's modulus, very low elasticity, and a hard, brittle surface. A few of the more commonly used ceramics are described here.

(a) Alumina

Alumina (Al_2O_3) is often used as coatings for metallic implants. The coating aids in tissue fixation of the implant by providing a porous surface for the surrounding tissue to grow into and mechanically interlock [49].

(b) Hydroxyapatite

Hydroxyapatite (HA) is the major mineral component of bone and teeth, with a chemical formula of $\text{Ca}_{10}(\text{PO}_4)_6$ [50]. It can be added to implants to encourage bone ingrowth and reinforce the implants as they begin to age [51].

(c) β - tricalcium phosphate

β -tricalcium phosphate (β -TCP) is represented by the chemical formula $\text{Ca}_3(\text{PO}_4)_2$, the Ca/P ratio being 1.5. TCP exhibits nontoxicity to tissues, bioresorption and osteoinductive property [52]. The main application of β -TCP is in bone tissue engineering [53].

2.3.4 Advantages of natural polymers

Among the presented biomaterials, polymers have been extensively explored in the area of tissue engineering. In general, natural biomaterials are more advantageous because of their inherent properties of biological recognition, including presentation of receptor-binding ligands and susceptibility to cell-triggered proteolytic degradation and remodelling [54].

Collagen was selected in this work based on its excellent biocompatibility and suitable interaction with cells and other biomolecules. Collagen scaffolds possess requisite hemostatic properties, low antigenicity, and appropriate mechanical characteristics for use in tissue engineering applications [55]. Furthermore, it will not decrease the local pH during degradation as compared to PLA and PGA that produce fragments of acid. Additionally, collagen scaffolds have been observed to promote cell and tissue attachment and growth [55]. It is suitable for use in both hard and soft tissue regeneration [43, 44, 56].

Apart from the choice of material, the desirable characteristics of a successful scaffold also include: suitable macrostructure, pore geometry, pore size, and physiochemistry properties. These characteristics were analyzed and the challenges were reviewed critically. The detail discussions were presented in Section 2.4.

2.4 Critical Issues and Challenges in Tissue Engineering Scaffold

There are several challenges in TE scaffold technology from both biological and engineering perspectives. Critical issues and challenges in TE scaffold are:

- Choice of biomaterial
- Effect of porosity
- Effect of pore size
- Effect of pore interconnectivity
- Effect of degradation rate
- Bioactivity of scaffold
- Consistency of processing method

Each of the issues is discussed in the following subsections.

2.4.1 Choice of biomaterial

The selection of the scaffold material plays a key role in the design and development of a particular TE product. Although the classical selection criteria focus on a safe, stable implant, it is recognized that every material used will elicit different cellular responses in terms of degradation [57]. One of the current challenges in making a cell-scaffold construct is to select a polymer scaffold that meets the mechanical properties and degradation times required.

The ideal polymer for a typical TE application should be configured such that it possesses the following properties [58]:

- Appropriate mechanical strength to mimic *in vivo* conditions.
- Rate of matrix regeneration close to biodegradability rate of the biomaterial.
- Does not invoke any inflammatory or toxic response.
- Is easily processable into the final product form, either porous or compatible with a range of extremely hydrophilic additives (starch, salt) to create porosity.
- Demonstrates acceptable shelf life and is easily sterilized.

2.4.2 Effect of porosity

A TE scaffold requires a highly porous structure (>90%) to achieve a large surface area. Bose and Bignon [59, 60] have shown that the increase of porosity is accompanied by an increment in cell growth. They suggested that a higher cell proliferation for samples with higher pore volume is primarily due to the effect of higher surface area per unit volume. However, the strength of the structure is compromised for the lost of volume to porosity. Hence, the requirement of TES often results in conflicting design demands.

Hollister *et al* performed a study using an image-based homogenization approach to optimize the scaffold microstructure and its effective stiffness [61]. The optimization problem was modeled with two objective functions namely, (1) Stiffness design, with the stiffness as the primary goal while maintaining a base level of porosity, and (2) Porosity design, where the primary goal is porosity with a base level of stiffness.

Pores in this model consisted of interconnecting orthogonal cylinders (see Figure 2.2). Their validation model was based on mandibular condyle trabecular bone and it was found that the result showed excellent agreement between native bone properties and designed scaffold properties.

However, only two time-points were considered in the model namely the initial time immediately after implantation and the time where the scaffold had degraded completely and only the regenerated tissue existed. The occurrence in between the two distinct time points was not considered. Nevertheless, this effort demonstrated the significance of porosity as one of the key scaffold properties that could influence the success of TE treatments.

Figure 2.2: Modelling in scaffold optimization [61].

2.4.3 Effect of pore size

Research has shown that cells do exhibit selectivity on pore size according to specific cell types. Lu *et al* performed a study on bone tissue engineering [62]. Their results showed that human osteoblasts can penetrate interconnections over 20 μ m in size, colonize and proliferate inside the macropores, but the most favourable size is over 40 μ m. The critical pore size is said to be greater than 150 μ m to favour new bone formation. Earlier research tested on PLGA scaffold with different pore sizes ranging from 150-710 μ m showed that stromal osteoblast cells were able to proliferate and differentiate over this range of pore size [24]. However, these figures do not represent a definite threshold limit.

Itala *et al* used a highly standardized model to re-evaluate the threshold pore size of 100 μ m for bone growth under a non-load-bearing condition [63]. They found that the amount of bone growth was independent of the pore size and the implant thickness since there was formation of osteonal bone structure even in the smallest pore size of less than 75 μ m. However, the study did not examine the mechanical strength of the bone ingrowth. On the other hand, Holly *et al* reported a different concept that bone reconstruction would only be achieved by having a 3-D temporary matrix with a large macroporous interconnected structure with pore size ranging from 1.2 to 2.0mm [64]. Therefore, more knowledge input from the physiological aspect is necessary to establish more specific requirements.

Zeltinger *et al* performed a study on PLLA scaffold with different pore size using canine dermal fibroblasts, vascular smooth muscle cells and microvascular epithelial cells [65]. They found that canine dermal fibroblasts was indifferent to pore size while vascular smooth muscle cells and microvascular epithelial cells favoured a pore size of 90 μ m and greater than 107 μ m respectively. This result suggests the existence of a preferred pore size for different type of cells. However, no definite value for a preferred pore size to a specific cell type was found.

Therefore, the preferred pore size is expressed in a range of value instead of a specific figure as shown in Table 2.2.

Cell/ Tissue types	Preferred Pore Size
Fibroblast ingrowth	5-15 μm
Regeneration of adult mammalian skin	20-125 μm
Osteoid ingrowth	40-100 μm [66]
Regeneration of bone	100-350 μm [67], 100-600 μm [68]
Fibrovascular	> 500 μm [69]

Table 2.2: Guidelines for pore size.

In terms of the scaffold's mechanical properties, the pore size plays a less dominal role in comparison to the pore volume [59]. Pore size has been observed to influence adhesion, growth and differentiation of a wide variety of cell types.

Ranucci *et al* studied the differentiation of liver cells on collagen scaffold at 3 levels of pore size namely subcellular level (10 μm), cellular level (18 μm) and supercellular level (82 μm) [70]. The results showed that at subcellular level, a cell was restricted to grow in 2-D only and the function of the cell achieved was 30% less than a normal liver cell. Pore sizes at the cellular level were found to strongly impact the differentiation behaviour of the cell. Liver cell switched from differentiation to growth and hence, no function was achieved. At 82 μm pore size, which was much bigger compared to a individual cell, differentiation of liver cell was observed. Cell growth was not restricted in 2-D and 3-D directions, which was likely to enhance the cell-cell and cell-matrix interaction. It was concluded that collagen pore size could directly regulate the cellular spreading behaviour.

Scaffold produced using conventional method exerts limited control over pore size. Pores are formed based on the leaching of soluble particulates. The pore size is decided by the morphology of the particles used. Particle size comes in a range rather than a precise uniform value. Limitation of pore generator includes randomly distributed and non-precise controlled of pore size with unreliable interconnectivity.

2.4.4 Effect of pore interconnectivity

Interconnectivity between pores is important in promoting cell ingrowth and migration to the core of the implant. It serves as a link or pathway between pores for nutritional elements and extracellular fluid circulation. Therefore, high interconnectivity can potentially increase vascularization, which is critical to ensure the survival of cells while colonizing the depth of the scaffold.

Cells are found to spread across the interconnection, connecting 2 macropores while growing into the scaffold in the experiment conducted by Lu *et al* [62]. Bignon also reported that more than the size of the macropores, the density of interconnections determines the colonization rate [59]. Lu *et al* showed that 50 μm of interconnection size is needed to favour bone growth inside the pores. They argued that porosity and pore interconnection density are more important than pore size.

In recent development, researchers have been producing scaffolds with interconnected channels so as to simulate the vascular bed in natural tissue. Channels ranged from 135 μm to 500 μm were produced [71]. Such resolution is easily achieved using RP to fabricate the scaffold. The lower limit of the resolution is dependent on the nature of the RP process.

2.4.5 Effect of rate of degradation

In an ideal case, the scaffold should be remodelled and resorbed by growing cells and gradually replaced by the newly formed extracellular matrix and differentiated cells. A desirable feature would be the synchronization of the polymer degradation rate with the rate of tissue ingrowth. Therefore, the degradation properties of a scaffold are crucial to the success of the scaffold-based approach. There are two types of biodegradation and both are discussed in this section [72].

Polymer's degradation by a simple chemical hydrolysis of the backbone of the polymer occurs in two phases. In the first phase, water penetrates the bulk of the device, attacking the chemical bonds and converting long polymer chains into shorter fragments. This occurs in the amorphous phase and initially there is a reduction in molecular weight without a loss in physical properties. The reduction in molecular

weight is followed by a reduction in physical properties, as water begins to cause fragmentation to the device. Fragments that have a molecular weight below 50,000 are filtered and excreted by the kidney without being fully metabolised.

In the second phase, enzymatic attack and metabolization of the fragments occurs, resulting in a rapid loss of polymer mass. This type of degradation, where the rate at which water penetrates the device exceeds that at which the polymer is converted into water-soluble materials, is called bulk erosion. This results in erosion throughout the device. All commercially available synthetic devices and sutures degrade by bulk erosion.

A second type of biodegradation, known as surface erosion, occurs when the rate at which the water penetrates the scaffold is slower than the rate of conversion of the polymer into water-soluble materials. Surface erosion results in the device thinning over time while maintaining its bulk integrity. This is favourable as the erosion process is predictable and the size of the material might remain constant for a considerable period of time during its application.

The degradation-absorption mechanism is the result of many interrelated factors that include hydrophilicity of the polymer backbone, number of reactive hydrolytic groups in the backbone, degree of crystallinity, presence of catalysts, additives, impurities, or plasticizers, volume of porosity and the amount of surface area.

Balancing each of these factors will allow an implant to slowly degrade and transfer stress at an appropriate rate to surrounding tissues as they heal. This is one of the major challenges facing TE research today.

Effort has been made to model the polymer degradation and erosion so as to predict the pH changes on the surface to make sure it is compatible to the cell in TE. The erosion is assumed to be a Poisson process in Monte Carlo model under Random theory [73]. Parameters included are shape, crystallinity, porosity and tortuosity of the material. This theory is expanded to diffusion theory that takes into account the release of oligomer.

2.4.6 Bioactivity of scaffold

The interaction of cells on the scaffold is governed by both structural and chemical signalling molecules that play a decisive role for cell adhesion and further behaviour of cells after initial contact [74]. Figure 2.3 shows the integrin-mediated adhesion mechanism in cells.

Figure 2.3: Integrin-mediated adhesion in cell [75] .

The extent of initial cell adhesion determines the number, size, shape and distribution of focal adhesion plaques formed on the cell membrane, which subsequently describe the size, and shape of the cell spreading area. The extent of spreading is crucial for further migratory, proliferation and differentiation behaviour of anchorage dependent cells.

Current strategies to control the proliferation and other behaviour of cells on advanced biospecific materials is by patterning the material surfaces with adhesive molecules or by incorporating and controlled release of biomolecules such as natural growth factors, hormones, enzymes or synthetic cell cycle regulators [74].

2.4.7 Consistent processing method

Notwithstanding some improvements attained, the control over scaffold architecture using many conventional techniques is highly process dependent rather than design driven as discussed in Section 2.2.2. As a result, RP is seen to be a viable alternative to achieve extensive and detailed control over scaffold architecture [11]. RP systems such as Fused Deposition Modelling (FDM), 3-Dimensional Printing (3-DP) and Selective Laser Sintering (SLS) have been proven to be feasible in producing porous structures for use in tissue engineering. RP methods are considered to be capable of precisely controlling pore size, pore geometry, pore interconnectivity, spatial distribution of pores and construction of internal channels within the scaffold.

A state-of-the-art review is presented in the following section on the application of RP in tissue engineering. The capabilities of each technique in fabricating the scaffolds are discussed with example.

2.5 Rapid Prototyping in Tissue Engineering

Rapid prototyping (RP) is a common name for a group of techniques that can generate physical model directly from computer-aided design data. It is an additive process in which a part is constructed in a layer-by-layer manner. In the direct fabrication method, researchers tailor the manufacturing technique to be tissue-engineering specific by three approaches, namely designing new biomaterial that fits the processing window of the commercial RP systems, modifying the commercial system to tolerate non-standard material or developing new RP systems that use biomaterials directly. In the indirect fabrication, a sacrificial mould method is typically employed to expand the material opportunity in scaffold fabrication using RP techniques. Further discussion on both approaches is presented in the subsequent sections.

2.5.1 Melt /dissolution deposition techniques

In a typical melt-dissolution deposition system, each layer is created by extrusion of a strand of material through an orifice while it moves across the plane of the layer cross section. The material cools, solidifying itself and to the previous layer [76]. A

representative system using melt deposition is the Fused Deposition Modelling (FDM).

(a) Fused Deposition Modelling

In this method, filament of materials are fed and melted inside a heated liquefier before being extruded through a nozzle. The system operates under a temperature-controlled environment. Researchers have demonstrated the feasibility of utilizing FDM to fabricate functional scaffold directly. Zein *et al* fabricated PCL scaffolds with honeycomb structure and channels size of 160-770 μ m at porosity of 48-77% [77]. Another group of researchers has successfully extruded scaffold made of polypropylene-tricalcium phosphate [78]. In a recent effort, human mesenchymal progenitor cells (hMPCs) were seeded on PCL and PCL-HA scaffolds fabricated by FDM [79]. Proliferation of cell toward and onto the scaffolds surfaces was detected. Drawbacks of the FDM technique include the need for input material at specific diametric size and material properties to feed through the rollers and nozzle. The high operating temperature restrains the biomimetic aspects of the scaffold produced. Moreover, the material deposited solidifies into dense filaments, blocking the formation of microporosity. Microporosity is an important factor in encouraging neo-vascularization and cell attachment [80]. In terms of mechanical-chemical signal, it is a pivotal parameter in cell signalling pathway.

Modifications of FDM to circumvent these limitations have encouraged the emergence of several new techniques. Some variants of FDM process include 3D fibre-deposition technique [81, 82], precision extruding deposition [83] and precise extrusion manufacturing [84].

(b) 3D Fibre-deposition Technique

In this method, the feedstock material is in pellet or granule form that can be poured into the heated liquefier directly. Poly(ethylene glycol)-terephthalate-poly(butylene terephthalate) (PEGT/PBT) block co-polymer scaffolds were fabricated for articulate tissue engineering applications [81]. Scaffolds with pore size gradient were produced using this technique [82].

(c) Precision Extruding Deposition (PED)

The extruder of this system is equipped with a built-in heating unit to melt the feedstock material. PCL scaffold with pore size of 250 μ m was fabricated [83].

(d) Precise Extrusion Manufacturing (PEM)

PLLA scaffold with controllable porous architectures from 200 to 500 μ m in size was produced with this method [84].

Melt process is generally undesirable in scaffold fabrication because of the elevated temperature involved in melting the material. This limitation drives the researchers to replace the melting process with a dissolution process of material. Systems developed include low-temperature deposition manufacturing [85], multi-nozzle deposition manufacturing [86], microsyringe based deposition system [87] and robocasting [88].

(e) Low-temperature Deposition Manufacturing (LDM)

The scaffold building cycle is performed in a low temperature environment under 0°C [85]. A PLLA/TCP pipe scaffold has been produced.

(f) Multi-nozzle Deposition Manufacturing (MDM)

This is an improved version of LDM with more jetting nozzles in the system. In the work by Yan *et al*, biomolecules bone morphogenic protein (BMP) was suspended in water and being sprayed into the scaffold during its forming process [86].

(g) Pressure Assisted Microsyringe (PAM)

A microsyringe is used to expel a dissolved polymer under low and constant pressure [87]. Vozzi *et al* developed PCL and PLLA scaffolds with line width of 20 μ m [87]. Investigators also demonstrated that the performance of this method was comparable to soft lithography [89].

(h) Robocasting

This patented system is able to lay down highly concentrated colloidal suspension that is pseudoplastic-like [88]. Therriault *et al* fabricated 3-D microvascular network by

robocasting fugitive organic ink, followed by scaffold infiltration with epoxy resin and further post processing [90].

In general, the scaffolds fabricated using melt or solution deposition techniques described previously usually serve as hard tissue scaffolds. A group of researchers has developed an aqueous system, 3-D bioplotter [91], to meet the demand for fabrication of hydrogel scaffolds useful in soft tissue engineering. Hydrogels are becoming an increasingly important material for tissue engineering because of the high water content and mechanical properties which are similar to those of soft tissue in the body. Ang *et al* adopted a similar concept to develop a robotic dispenser, RPBOD, for fabrication of chitosan scaffold [92].

(i) 3D Bioplotter

The key feature of this method is the 3-D dispensing of liquids and pastes in a liquid medium with matched density [91]. The liquid medium compensates for the gravitational force and hence no support structure is needed. Hydrogel scaffolds with well-defined internal pore structure were prepared by Landers *et al* [93]. The hydrogel scaffold had interconnected pore at the range of 200-400 μ m.

(j) Rapid prototyping robotic dispensing system (RPBOD)

This system, developed by Ang *et al*, consists of a computer-guided desktop robot and a one-component pneumatic dispenser [92]. Material in liquid form was dispensed into a dispensing medium through a small Teflon-lined nozzle. Chitosan scaffolds with pore size of 400-1000 μ m were produced in the preliminary study.

2.5.2 Particle bonding techniques

In particle bonding techniques, particles are selectively bonded in a thin layer of powder material. The thin 2-D layers are bonded one upon another to form a complex 3-D solid object. During fabrication, the object is supported by and embedded in unprocessed powder. Therefore this technique permits the fabrication of through channels and overhanging features. After completion of all layers, the object is removed from the bed of unbonded powder. Typical systems in this technique are 3DPTM [94-96], TheriFormTM [97] and SLS [98-100].

(a) 3-Dimensional Printing (3DP™)

In this process, a stream of adhesive droplets is expelled, selectively bonding a thin layer of powder particles to form a solid shape. Kim *et al* [94] employed 3DP™ with a particulate leaching technique for creating porous scaffolds using PLGA mix with salt particle and a suitable organic solvent.

In an effort to render the system more biocompatible, Lam *et al* have formulated a blend of starch-based polymer powders that can be bonded using distilled water [95]. Another group used water-based binder to produce porous hydrophobic polyethylene parts [96]. Binder was printed onto the mixture of polyethylene and water-soluble adhesive powder bed.

(b) TheriForm™

This commercial system is similar to 3DP™ where a printhead assembly deposits binder droplets onto selected regions of the powder, swelling and dissolving the polymer powder in the printed regions [97]. Porous sintered HA bone repair scaffolds were fabricated using the TheriForm™ process [101].

(c) Selective Laser Sintering (SLS)

The machine uses a deflected CO₂ laser beam to selectively scan over the powder surface. It is the preferred forming process to produce complex porous ceramic matrices suitable for implantation in a bone defect as demonstrated by Vail *et al* [99]. Tan *et al* has successfully sintered polyetheretherketone-hydroxyapatite (PEEK/HA) powder blends on a commercial SLS machine [100].

2.5.3 Indirect RP fabrication method

RP systems can also be utilized to produce a sacrificial mould to fabricate tissue-engineering scaffolds. These multi-step methods usually involve casting material in a mould and then removing or sacrificing the mould to obtain the final scaffold. Such techniques allow the user to control both the external and also the internal morphology of the final construct. In addition, indirect methods also require less raw scaffold material while enabling increased material opportunities and making it possible to use a composite blend that may require conflicting processing demands.

This method also enables the processing of thermal-sensitive biomaterial since the original properties of the biomaterial are well-conserved as no heating process is imposed on the scaffold material. Common RP systems employed are MMIITM [71, 102-106], SLA [107, 108] and FDM [60].

(a) ModelMakerIITM (MMII)

This machine uses a single jet each for a plastic build material and a wax-like support material. The printer head ejects droplets of the materials as they are moved in a horizontal X-Y fashion. A milling head is passed over each layer to ensure that a uniform thickness has been achieved.

Taboas *et al* produced PLLA scaffold with local and global porosity for bone tissue engineering, specifically trabecular bone [102]. Limpanuphap and Derby fabricated TCP scaffolds with controlled internal porosity with a suspension of TCP in acrylate binder [103]. A similar route was used to produce polymer/TCP scaffold, which is believed to show more potential for cell adhesion. Wilson *et al* fabricated and characterized HA scaffolds with a defined macro-architecture [104]. Sachlos *et al* have successfully produced collagen scaffolds with predefined and reproducible internal channel [71]. The smallest channel width achieved was reported to be 135µm. Similar processing steps were adopted by Manjubala *et al* to produce composite scaffolds of chitosan and hydroxyapatite [105].

Recent studies have reported the fabrication of porous PCL scaffold by using a 3D inkjet printed thermoplastic mould [106]. Composites of PCL and calcium phosphate were subsequently fabricated by injection moulding of molten polymer-ceramic composites through the printed mould.

(b) Stereolithography (SLA)

In this system, a UV laser traces out the layer on the surface of a vat of photocurable resin, solidifying the model's cross-section while leaving non-part areas liquid. The elevator then drops enough to cover the solid polymer with another layer of liquid resin and the laser traces the second layer atop the first. Chu and Halloran have produced HA-based porous implants using SLA built epoxy moulds [107]. A similar

group of investigators carried out an *in vivo* study using two different architectural designs, orthogonal and radial channels [108]. Preliminary results showed that controlling the overall geometry of the regenerated bone tissue was possible through the internal architectural design of the scaffolds.

(c) FDM

Bose *et al*'s research aimed to investigate the effect of pore size and porosity on the mechanical and biological responses. Controlled porosity alumina and β -TCP ceramic scaffolds with pore sizes in the range of 300 to 500 μ m and pore volume of 25 to 45% were produced [60].

The strengths and weaknesses of all the techniques described in Section 2.5 are summarized in Table 2.3.

RP system	Resolution μm	Strength	Weakness	Reference
Melt/Dissolution Deposition Techniques				
Fused Deposition Modelling (FDM)	250	Good mechanical strength; Versatile in lay-down pattern design	High temperature; Need to produce filament material; narrow processing window; rigid filament	78
3D Fibre-deposition Technique	250	Input material in pellet form, preparation time is reduced	High temperature; rigid filament	81
Precision Extruding Deposition (PED)	250	Input material in pellet form;	High temperature; rigid filament	83
Precise Extrusion Manufacturing (PEM)	200-500	Input material in pellet form;	High temperature; rigid filament	84
Low-temperature Deposition Manufacturing (LDM)	400	Can incorporate biomolecule;	Solvent is used; required freeze drying	85
Multi-nozzle Deposition Manufacturing (MDM)	400	Enhanced material opportunity; Can incorporate biomolecule	Solvent is used; required freeze drying	86
Pressure Assisted Microsyringe (PAM)	10-600	Enhanced material opportunity; Can incorporate biomolecule; Very fine resolution can be achieved	Small nozzle inhibit incorporation of particle; narrow range of printable viscosities; Solvent is used	87
Robocasting	100-1000	Enhanced material opportunity;	Precise control of ink properties is crucial	88
3D Bioplotter	250	Enhanced material opportunity; Can incorporate biomolecule;	Low mechanical strength; Smooth surface; Low accuracy; Slow processing; precise control of material and medium properties; calibration for new material	91
Rapid Prototyping Robotic Dispensing System (RPBOD)	400-1000	Enhanced material opportunity; Can incorporate biomolecule;	Precise control of material and medium properties; required freeze drying	92

Table 2.3: Comparison of different rapid prototyping technologies applied in tissue engineering.

RP system	Resolution μm	Strength	Weakness	Reference
Particle Bonding Techniques				
3-Dimensional Printing™	200	Micropositivity induced in the part; Enhanced material opportunity; Water as binder; No support structure needed; Fast processing	Material must be in powder form; Limited mechanical strength; Powdery surface finish; Trapped powder issue; May required post processing	94
TheriForm™	300	Micropositivity induced in the part; Enhanced material opportunity; non- organic binder is possible; No support structure needed; Fast processing	Material must be in powder form; Powdery surface finish; Trapped powder issue	97
Selective Laser Sintering	500	Micropositivity induced in the part; Enhanced material opportunity; No support structure needed; Fast processing	Material must be in powder form; High temperature; Powdery surface finish; Trapped powder issue	98
Indirect RP Fabrication Method				
FDM	250	Enhanced material opportunity; Control of external and internal morphology;	Multi-steps involved	60
MMII	180	Enhanced material opportunity; Control of external and internal morphology;	Multi-steps involved	71
SLA	366	Enhanced material opportunity; Control of external and internal morphology;	Multi-steps involved	108

Table 2.3: Comparison of different rapid prototyping technologies applied in tissue engineering (continue).

2.6 Challenges of Rapid Prototyping in Tissue Engineering

RP holds great potential for the fabrication of scaffold. In spite of the increasing interest on the use of RP in TE, there are several challenges that need to be addressed, namely limited material opportunity, the optimal scaffold design, the bioactivity of the scaffold as well as the cell seeding and vascularization issues. These are illustrated in Table 2.4.

New material opportunity	Design of scaffold	Bioactivity of the scaffold	Cell seeding and vascularization of scaffold
<ul style="list-style-type: none">• Material Preparation• Degradation Product• Mechanical Strength of Scaffold	<ul style="list-style-type: none">• Scaffold Morphology• Surface Topography	<ul style="list-style-type: none">• Surface modification of 3-D structure• Incorporation of biomolecules	<ul style="list-style-type: none">• Bioreactor technology

Table 2.4: Challenges of RP in TE scaffold.

Each of the issues in Table 2.4 is discussed in detail in the following sections.

2.6.1 New material opportunity

RP techniques use various technologies that range from laser scanning to printing technology as described in Section 2.5. Each technique is highly specific in that only a certain type and form of material can be processed on each system, rendering the RP systems to have limited choices of material or material opportunity. The following issues such as material preparation, degradation product and the mechanical strength of scaffold need to be addressed when exploring non-standard material for TE applications.

(a) Material Preparation

RP techniques are very specialized technologies in term of material processability. Each technique requires a specific form of input material such as filament, powder, solid pellet or solution. Therefore, the choice of material for the scaffold must be compatible with the selected RP process and be efficiently produced into the form required. Other considerations during the selection of materials include the degradation profile, the mechanical strength and other properties of the scaffold.

(b) Degradation Product

Even though degradation products of biodegradable polymers are known to be largely non-cytotoxic, little information is available regarding the degradation rate-dependent acidic by-product effects of the scaffold. Sung *et al* found that fast degradation of the polymer negatively affected cell viability and migration into the scaffold *in vitro* and *in vivo* [109]. This can be explained by the change of rapid local pH due to the polymer degradation. Therefore, a more systematic investigation approach is needed to classify the material degradation profile.

(c) Mechanical Strength of Scaffold

Cells are able to detect very sensitively the mechanical properties of the adhesion substrate and regulate the integrin binding, assembly of focal adhesion plaques and cytoskeleton accordingly [110]. If the adhesion substrate is too rigid and non-deformable, the cells are not able to reorganize and recruit the receptors into focal adhesion plaques, which is a prerequisite for delivery of signals ensuring the viability of anchorage-dependent cells. Similarly, if the material is too compliant, it does not allow the anchorage of cells due to the inability of resisting the tractional forces generated by the assembling cytoskeleton. More detail and controlled experiments are necessary to further characterize the impact of material compliancy on the behaviour of cells.

2.6.2 Design of scaffold

RP systems such as FDM are able to produce scaffold with designed morphology by manipulating the scaffold material directly through the numerical-mechanical control systems. Filaments of material are deposited at predetermined location as specified in the input design drawing, generating different configuration of interconnected channels (see Figure 2.4). The channels are fully interconnected with reproducible size and controlled distribution.

Figure 2.4: Different channels design of scaffold [77].

However, the scaffold produced is limited to synthetic polymer only. The impact of the design of a scaffold on the selectivity behaviour of cells has not been studied systematically. Furthermore, there are few more issues to be considered such as the morphology and the surface topology of the scaffold.

(a) Scaffold Morphology

RP fabricated scaffold generally presents many edges and grooves. For example, in a scaffold fabricated by FDM, the filaments of deposited material are at an angle to the layer beneath it, creating a discontinuity in structure (see Figure 2.5).

Figure 2.5: Deposited filaments by FDM.

These discontinuities in topography can affect the adhesion and migration of cell as shown by Yin's work in 2004. Yin *et al* grew cardiac cells on microgrooved elastic scaffolds to investigate the topography-driven changes in cardiac electromechanics [111]. The grooves are 50 μm in depth and 120 μm in width. They demonstrated direct influence of the microstructure on cardiac function and susceptibility to arrhythmias via calcium-dependent mechanisms.

(b) Surface Topography

Topography features have been shown to orientate cells in a phenomenon known as “contact guidance” [112]. The surface roughness of the scaffold is important in cell-matrix interaction. The rough powder surface produced from powder based RP technique may enhance cell adhesion (see Figure 2.6).

Figure 2.6: Surface of powder.

However, if the surface is too rough, the cells adhering to these materials may not be able to develop distinct focal adhesion plaques or bridge over the irregularities. Besides, the sharpness of the surface might damage the cell physically. In certain RP systems such as FDM and bioplotter, the smooth surface of solidified material cannot ensure firm cell adhesion and therefore require further surface modification or coating. The impact of surface roughness of the RP-fabricated scaffolds should be investigated in detail.

2.6.3 Bioactivity of the scaffold

Incorporating biomolecules can enhance the bioactivity of the scaffold. The biomolecules can be coated onto the surface or by integrating the biomolecules into the bulk material. Some RP systems that have excluded high temperature operation such as MDM and bioplotter offer the opportunity of incorporating the biomolecule during part building cycle. However, further information such as the types of biomolecule, the optimal concentration and spatial control of these biomolecules is needed to produce a highly desirable scaffold.

2.6.4 Cell seeding and vascularization of scaffold

One significant challenge in scaffold based TE approach is to distribute a high density of cells efficiently and appropriately throughout the scaffold volume. Currently, the only FDA approved cell seeding process is by using the petri dish. However, this method fails to deliver cell deep inside the scaffold with uniform distribution [113-115]. Therefore, the cellularization of a 3D scaffold is closely related to the advances of bioreactor technologies.

Bioreactors are generally defined as devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled operating conditions [12]. The types of bioreactor currently available include spinner flask, perfusion cartridge and rotary cell culture system [20]. Each of the systems utilizes different physical principles and may necessitate specific design consideration on the scaffold shape and strength. Further discussion on bioreactor is presented in Section 2.7.

RP systems present great flexibility in scaffold design and development. RP fabricated scaffold can be designed to have interconnected flow channels to fit into the operation of the bioreactor as displayed by the work of Sakai *et al* [116] (see Figure 2.7).

Figure 2.7: Flow channels in scaffold [116].

Sufficient vascularization of the scaffold to maintain adequate perfusion is a primary consideration in the engineering of large tissue constructs. Rapid and high level of vascularization of the cell-seeded scaffold is essential to meet the challenge.

One possible approach to achieve vascularization is by incorporating growth factors into the scaffold. A number of angiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet-derived growth factors (PDGFs) and transforming growth factors (TGFs), have been identified to promote the formation of new vascular beds from endothelial cells present within tissues [117]. Sheritan *et al* believed that the localization and control release of these factors from a matrix might allow an enhanced vascularization of engineered tissues [118].

An alternative approach to enhance the rate of vascularization is to transplant endothelial cells on scaffold [119]. Experimental studies with rats have confirmed that the vascularization of matrices is accelerated with endothelial cell transplantation. The bioactivity of the scaffold plays a crucial role in this approach.

2.7 Bioreactor in Tissue Engineering

Conventional static cultures of 3-D cell-scaffold constructs were found to be inadequate in several aspects. For example, PLGA constructs cultivated with stromal osteoblasts in static cultures had inhomogeneous cell and matrix distribution throughout the scaffolds [24]. The maximum penetration depth of mineralized matrix was reported to be 240 μm from the top surface of the scaffold even after 56 days of cultivation. This was due to the fact that such cultures induced a thin layer of cells to cover the exterior of the scaffolds while not penetrating into the scaffolds.

On the other hand, in cultures where dynamic conditions exist, much improved effects were observed. Physical forces created by the hydrodynamic conditions such as fluid flow-induced shear stress and pressure were found to enhance cellular and matrix distributions [20, 21]. Other effects such as improved cell proliferation and increased mineralized matrix deposition were also observed [20]. It has become clear that physical and mechanical stimuli are extremely important in scaffold-based tissue engineering.

This has motivated researchers to utilize bioreactors. Bioreactors are generally defined as devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions (e.g. pH, temperature, pressure, nutrient supply and waste removal) [12]. Functions of bioreactors include at least one of the following: (1) promote uniform cell distribution in 3D tissue engineered scaffolds, (2) maintain desirable gas and nutrients concentrations in the culture medium, (3) cater efficient mass transfer to the tissue being constructed and, (4) apply controlled mechanical stimuli (such as dynamic compression and cyclic strains) on the tissue constructs.

Some representative bioreactors for tissue engineering applications include spinner-flasks [13], rotating-wall vessels [14], hollow-fibre bioreactors [15], direct perfusion bioreactors [16-18] and bioreactors that apply controlled mechanical forces such as dynamic compression and cyclic strains [19].

2.7.1 Spinning flask bioreactor

The spinner-flask bioreactor is a simple cylindrical glass container in which growing tissues are suspended and a stirring element located at the bottom of the tank ensures the mixing of the culture medium [13] (see Figure 2.8). In a study, rat marrow stromal cells were seeded on PLGA scaffolds and cultured under static or in a spinner flask [120]. Results obtained showed that the spinner flask culture demonstrated a 60% enhanced proliferation at the end of the first week when compared to static culture.

Figure 2.8: Spinner-flask bioreactors [12].

2.7.2 Rotating wall bioreactor

Several groups of researchers have developed rotating bioreactors in three different forms, namely, the slow-turning lateral vessel (STLV), the high-aspect ratio vessel (HARV) and the rotating-wall perfuse vessel (RWPV) (see Figure 2.9) [14].

Figure 2.9: Rotating wall bioreactors [121].

These vessels provide a dynamic culture environment to the constructs, with low shear stresses and high mass-transfer rates. Another group of researchers have also proved that rotating vessels can provide the dynamic laminar flow patterns required for seeding and in vitro cultivation [122]. These improved the structural and

functional construct properties of the scaffolds. A recent study reported the use of rotating bioreactor to engineer temporomandibular joint disc [123].

2.7.3 Hollow fibre bioreactor

These bioreactors can be used to enhance mass transfer during the culturing of highly metabolic and sensitive cell types such as hepatocytes [15]. In one configuration, cells are embedded within a gel inside the lumen of permeable hollow fibres and a medium is perfused over the exterior surface of the fibres [12] (see Figure 2.10).

Figure 2.10: Hollow-fibre bioreactor [12].

2.7.4 Direct Perfusion bioreactor

In a direct perfusion bioreactor, the culture medium is perfused directly through the pores of the scaffolds. The success of this design is a careful balance between the mass transfer of nutrients and waste products to and from cells, the retention of newly synthesized extracellular matrix components within the construct, and the fluid-induced shear stresses within the scaffold pores [12]. Direct perfusion bioreactor had been reported on the cultivation of vascular smooth muscle cells [124], bone marrow stromal cells [17], and rat hepatocyte spheroids [125].

2.7.5 Mechanical stimuli bioreactor

These bioreactors apply controlled mechanical forces such as dynamic compression to engineered constructs (see Figure 2.11). They can be used as modelling of tissue development under physiological loading conditions, and to generate functional tissue grafts. Compressive deformation can be applied by computer-controlled micro-stepper motor, and the stress on the constructs can be measured using a load cell [12].

Figure 2.11: Bioreactor providing mechanical forces [12].

2.8 Challenges of Bioreactor in Tissue Engineering

In spite of the increasing interest in the use of bioreactor in TE, there are several challenges that need to be addressed, namely the design issues, the physiological parameters involved and the mathematical modelling of the bioreactors.

2.8.1 Design of bioreactors

Bioreactor design in tissue engineering is very complex and is still at an early stage of development [126]. Current use of bioreactor mainly accentuates in the area of research and only a few have been commercialized. This could be due to the fact that each bioreactor is highly customized to accommodate the types of scaffold used in terms of the scaffold material, mechanical properties, overall size and the type of cells cultured. Apart from that, the bioreactor design must be synchronized to the objective of the investigation.

(a) Main function of the bioreactor

The functions of the bioreactor vary from case to case. In many studies, rotating and perfusion bioreactors are adopted with the objective of achieving maximum cell proliferation rate. The design function of these bioreactors is to enhance the mass transport mechanism through the scaffolds. In other design, the main function of the bioreactor is to apply hydrostatic pressure.

Mizuno *et al* had hypothesized that applied hydrostatic fluid pressure would stimulate chondrogenesis of chondrocytes in culture [127]. A pressurized culture system incorporating a pressure-proof culture chamber and backpressure regulator was designed for the application of positive pressure of 2.8MPa. On the other hand, a low-pressure system was used to facilitate the perfusion of larger number of bone marrow stromal cells into the porous ceramic scaffold and hence, enhancing the bone formation within the scaffolds [128]. A bioreactor with added functions was recently reported whereby it was operated in an automated manner for successive culture of anchorage-dependent cells [129]. In summary, the specific aim of the bioreactor must be defined clearly and the design should directly support it.

(b) Control and monitor of the bioreactor

A bioreactor is an *in vitro* device that tries to mimic the *in vivo* biomechanical and biochemical environment for functional tissue growth. Therefore, important physiochemical parameters such as pH, oxygen tension, carbon dioxide tension, and temperature should be tightly controlled. Such a control system requires both hardware and software solutions. The bioreactor system should ideally incorporate flow through electrodes for easy monitoring of the parameters. The monitoring system selected should be able to interface with a common working platform for data recording and analysis. In certain designs, sampling ports are incorporated in the bioreactor system for regular collection of culture medium samples [130]. The samples are then analyzed using a blood analyzer to monitor the metabolic activities of the cells in cultivation.

2.8.2 Physiological parameters input

Several important physiological parameters in the design of bioreactor were investigated, namely (a) dissolved oxygen tension, (b) fluid shear stress, and (c) hydrostatic pressure.

(a) Dissolved oxygen tension

Dissolved oxygen tension (DOT) is an important physiology parameter for cell growth. A study was performed on the metabolic activity of a hybridoma population immobilized in macroporous collagen microspheres [131]. The data obtained showed a reduction of the metabolic activity at reduced DOT. Domm *et al* found that low

oxygen tension promoted redifferentiation of dedifferentiated bovine articular chondrocytes cells in alginate bead culture [132]. Another study showed that low oxygen tension enhanced chondroinduction by demineralized bone matrix in human dermal fibroblasts *in vitro* [133].

(b) Fluid shear stress

Slow interstitial flow is present in all vascularized tissues *in vivo* as an important microcirculatory component between blood and lymphatic capillaries [134]. Hence the cells are subjected to fluid flow induced shear stress.

Figure 2.12 shows a cross section of cortical bone and bone marrow with the fluid flowing in radial direction [135]. Preosteoclasts, osteoclasts, bone lining cells and osteocytes are subjected to the fluid flow.

Figure 2.12: Cross-section of cortical bone and bone marrow [135].

In many bioreactor designs, fluid flow is utilized to minimize the nutrient gradient across the scaffold as well as providing mechanical stress to the cells cultured. However, the optimal flow profiles have not yet been well documented. The flow can be delivered in a continuous cyclic fashion. Repeated application of shear stress was found to have stimulated late phenotypic markers of osteoblastic differentiation of bone marrow stromal cells in a manner that depends on the duration of stimulus [136].

However, the magnitude of shear stress applied must not be too large as animal cells are also sensitive to shear forces due to their relative large size and the absence of a protective cell wall [137]. Furthermore, the profile of the fluid shear stress changes

according to the input signal. The signal can be ramp flow, impulse flow, or stepped flow with different duty circles, all of which will affect the shear stress generated.

(c) Hydrostatic pressure

In a study, bovine articular chondrocytes were grown in porous three-dimensional collagen sponges, to which hydrostatic pressure was applied at 2.8 MPa for up to 15 days [138]. It was found that high pressure enhanced matrix synthesis and accumulation by bovine chondrocytes. In the process of deciding the imposed physiological parameters, the designer must be aware of the potential impact of these factors as well as setting the reasonable input values for these parameters.

2.8.3 Mathematical modelling of bioreactors

The hydrodynamics of the bioreactor is determined by the operating conditions such as the flow rate, mass-transfer rate, stress level imposed and flow profile. All these will affect the biochemical composition and the morphology of the resulting tissue. Current reviews reflect that the control of the hydrodynamics of bioreactors was achieved mostly by trial and error methods. Optimization of the parameter inputs experimentally could be time-consuming and labour-intensive. Therefore, mathematical and computational modelling of the bioreactor is necessary to describe the hydrodynamics of the bioreactor and to optimize the input parameters efficiently. There have been several reports on the modelling of oxygen gradient in scaffolds [130, 139, 140], flow through scaffolds [141-144] and hydrodynamics in bioreactors [145, 146].

(a) Modelling of oxygen gradient in scaffolds

There have been several mathematical models reported to describe the relationship between the bioreactor designs, the operating parameters used and the cell growth inside the bioreactors [130, 139, 140].

A model that described the oxygen concentration in the bulk medium layer and the cell layer was reported by Zhao *et al* [130]. The model used a volume-averaging method to obtain the continuity equation that described the oxygen profile in a two-phase region made up of cells and the medium. Metha *et al* reported a mathematical model to describe nutrient/growth factor transport and cell growth inside a

microchannel bioreactor [140]. Critical review on the models indicated that the equations were not a general solution as the basic equations were derived with different assumptions and the models were developed pertaining to the specific geometry of individual bioreactor.

(b) Modelling of flow through scaffolds

The modelling of fluid flow through porous scaffolds is a challenge due to the intricate and irregular pore network structures. The small scale of the scaffolds used, which is normally in the order of few millimeters, further complicates the modelling problem.

Flow through a PCL scaffold in a bi-axial rotating bioreactor was modelled by Singh *et al* [141]. The PCL scaffold's regular pore geometry made the modelling effort more attainable. In the case of a scaffold with irregular pore structures, micro-computed tomography imaging was used to define the scaffold micro architecture for simulations as reported by Porter *et al* and Cioffi *et al* [142, 144] (see Figure 2.13(a)).

Figure 2.13: Geometry modelling using micro-computed tomography imaging [144] and simplified assumption [143].

The analyzing techniques adopted in each of the models were problem-specific. Porter *et al* used the Lattice-Boltzmann method to simulate the flow conditions within perfused cell-seeded cylindrical scaffolds [142]. Cioffi *et al* modelled the micro-environment in polyestherurethane foam seeded with chondrocytes [144]. The results of the fluid dynamic simulations were analyzed at the central portions of the fluid domain to avoid boundary effects [144]. Boschett *et al* developed a computational fluid dynamic model of the flow of culture medium through a PLLA scaffold fabricated by particulate leaching method [143]. The geometry of the polymeric

scaffold was further simplified by assuming subtraction of a solid sphere from a concentric solid cube (see Figure 2.13(b)).

(c) Modelling of hydrodynamics in bioreactors

Ye *et al* reported a theoretical framework for hollow fibre bioreactors [145]. The influence of some important operating parameters, such as cell density, medium flow rate, cellular matrix thickness and hindering factors, on the nutrient concentration distribution in the bioreactor was analyzed using commercially available software, FEMLAB. Numerical characterization of the flow field within a spinner flask used to produce cartilage was reported [146].

Mathematical modelling and computational simulation are definitely valuable tools in the development of bioreactors. However, the formulation of the problem and the solution strategy adopted requires extensive knowledge in mathematics, fluid mechanics, mass transfer, as well as the skills in using the appropriate software simulation packages.

2.9 Summary

In summary, the emergence of various different approaches in tissue engineering ranging from scaffold-based approach to scaffold-free layer-by-layer manufacturing techniques highlighted the fact that the field tissue engineering is still growing. Looking towards the future, RP technologies hold great potential in the context of scaffold fabrication. The technology allows the tissue engineer to have full control over the design, fabrication and modelling of the scaffold being constructed, providing a systematic learning channel to investigate cell-matrix interaction.

RP techniques use several technologies ranging from laser to fusion of powder to printing technology. Each process is highly specific in that only a certain type and form of material can be processed on each system. This point is illustrated in Section 2.6.1. Corresponding to the specific material, the machine is calibrated to process the specific material only. Applying new or non-standard material to commercial RP machines always means that experiments must be conducted to re-calibrate or

optimize the process parameter settings. Instead of re-calibrating the machine parameters every time a new material is loaded, an indirect method that uses sacrificial mould provides an easier route to test new materials. Additionally, indirect RP methods coupled with conventional pore forming techniques significantly expand the material opportunity in TE. Furthermore, it is possible to use different material that may require different processing conditions or even conflicting processing conditions.

The objective of this research aims to overcome the challenges in processing collagen and to realize the design of porous collagen scaffold with 3-D internal channels. From the foregoing discussion, it is believed that the indirect method of fabrication deserves further exploration, and hence this approach is adopted in this research. The Solidscape 3-D inkjet printer (T612) is able to produce moulds using non-toxic thermoplastic and wax that can be removed by dissolution, rendering it to be the most suitable for indirect RP method.

Another pivotal element in scaffold-based tissue engineering is the strategy adopted to ensure uniform cellularity of the fabricated scaffold and to sustain high cell density in the scaffold. Bioreactors systems have been developed as a potential device to create *in vivo* biomechanical and biochemical conditions for functional tissue development. Different types of bioreactor have been described in many research articles.

Active research reported in this area has proved that the use of a bioreactor is an important strategy for continuous progress of scaffold-based tissue engineering. In this project, a direct perfusion bioreactor was designed and used for cultivation of the scaffold fabricated via indirect rapid prototyping technique. Direct perfusion bioreactors have the ability to enhance diffusion and apply mechanical forces on the construct at the same time. The perfusion of medium allows nutrients and oxygen to penetrate through the porous scaffolds and can bring about superior cell growth throughout the scaffolds. Further details on the design and development of the bioreactor are presented in Chapter 6.

CHAPTER 3

MODELLING OF 3-D INKJET PRINTING PROCESS

Ink-jet printing is an ever-evolving technology with expanding applications. It is one of the established techniques employed in rapid prototyping, especially in droplet-based manufacturing technique. However, such digital fabrication that involves small diameter high-speed droplet requires precise control of the droplet volume and momentum. This chapter presents an analysis to model the inkjet printing process, specifically the Solidscape 3-D inkjet printer (T612), to provide physical insight on the process. The analytical model is useful in the prediction of printing performance and investigation of the effect of specific variables. The machine's performance is important in defining the ability to achieve the predetermined morphology and resolution. Application of the parts printed is demonstrated.

3.1 Background on Inkjet Printing Technologies

Inkjet printing is one of the more important technologies in precise droplet deposition systems. It is a technology that has found its way in various industries outside the graphic art-printing field. Examples of applications include: biomedical and chemical sample handling [147-149], fuel mixing control [150, 151], direct writing and packaging [152-154], optical component fabrication [155, 156], microfabrication [157, 158], rapid prototyping [159-161] and manufacturing processes [162-165].

Inkjet print head can be operated using different actuation mechanisms such as pneumatic, thermal bubbles, electrostatic, piezoelectric, etc. The two most common inkjet technologies are continuous inkjet (CIJ) and drop-on-demand inkjet (DOD).

3.1.1 Continuous inkjet

In CIJ, ink is conductive. The ink is forced out of the nozzles by pressure (see Figure 3.1), forming a stream that breaks up into droplets by Rayleigh instability.

Figure 3.1: Continuous inkjet printing [166].

The continuously generated droplets pass through a charge plate to be charged and deflected to the printout. The charged drops can be steered by applying electrostatic field to control the size and spacing of the droplets. Drops not required for printing are captured and recirculated. The application of this device is limited since a complex system for droplet deflection and collection is required. The ink particle must also be able to conduct electricity and the recycling of unused droplets can induce contamination in the ink chamber.

3.1.2 Drop on demand inkjet

In this mode, droplets are generated when the printhead is actuated. The two dominant techniques for actuation are the thermal and piezoelectric DOD printing (see Figure 3.2).

Figure 3.2: Drop-on-demand inkjet printing [166].

In thermal DOD printing, liquid ink in the chamber is heated by pulse current applied to the heater under the chamber. The temperature of the ink rises to around the liquid critical point in microseconds and leads to the formation of a bubble on the surface of the heater. The bubble pushes liquid out of the nozzle to form a droplet. The bubble collapses when the heating pulse is turned off after the ejection of the droplet.

In piezoelectric DOD printing, ink drops are formed when required. There are four modes of operation namely bend mode, squeeze mode, push mode and shear mode (see Figure 3.3). The operational principle is based on generation of an acoustic wave in a fluid-filled chamber by a piezoelectric transducer via the application of a voltage pulse. The nozzle orifice serves as a capillary valve and droplet is ejected when the local pressure inside the chamber exceeds a critical value. Position control is achieved by mechanically placing the printer head above the desired location before droplet ejection. A print head using piezoelectric actuation has the advantage of easy control of drop size by adjusting the driving parameters. Exclusion of heat in this mode also ensures superb reliability and longer tooling life.

Figure 3.3: Piezoelectric operation.

3.2 Modelling of 3-D Inkjet Printing Process

3-D Inkjet Printing operates at the individual droplet size level. It is a variable mass process. Droplets are generated from a piezoelectric drop-on-demand printhead. The droplet impacts on the substrate and spreads into a spherical cap defining the resolution of the machine. At an appropriate printing condition, these droplets coalesce and make a straight line as the printhead transverses. The width of the line depends on the extent of droplet spreading. The lines join together and form a layer. Addition of multiple layers forms a part of desired shape. A schematic representation of the process is presented in Figure 3.4.

Figure 3.4: Schematic representation of droplet-based manufacturing.

Droplet based process physics involves a multitude of challenges in fluid mechanics, thermodynamics and heat transfer, with the coupling of piezoelectricity, elasticity, and free surface fluid dynamics.

One of the objectives of this study is to develop a simplified analytical model of the inkjet printing process, specifically to the T612 3-D inkjet printer (Solidscape, USA), so as to provide a simple insight and understand the fundamentals of the process. The analysis is useful in the prediction of the printing performance, material selection, optimum configuration of parameters and investigation of the effect of specific process variables.

The model is presented as a block diagram in Figure 3.5. The printing process is modelled in 3 levels, namely (1) the single droplet level, (2) successive droplets deposition and (3) the interaction of build-support material.

Material is stored in the material reservoir and transported to the printhead via two heated feedlines to the individual printhead. A driver that incorporates electrical and mechanical component actuates the piezoelectric sleeve in the printhead to initiate the propagation of acoustic waves in the ink chamber. The pressure wave at the nozzle exceeds the surface tension and ambient pressure, pushing the liquid at the nozzle to form a droplet. The droplets travel a short distance from the printhead and impact on the substrate directly below it. The impact energy causes the droplet to spread. Spreading is decoupled from solidification due to the very short time scale involved [169]. After spreading, the droplet loses its latent heat and solidifies into the solid phase.

At an appropriate printhead transverse speed and actuation frequency, the droplets are deposited at successive manner and coalesce to form a line before solidification. Subsequently the lines coalesce and form a layer.

Figure 3.5: Block diagram of 3D inkjet printing process.

3.2.1 Piezoelectric printhead basics

The inkjet printhead assembly is shown in Figure 3.6. It is a squeeze mode piezoelectric printhead consisting of a long channel with a nozzle [170]. The channel structure was designed to obtain a large incoming positive pressure wave at the nozzle.

Figure 3.6: 3-D inkjet piezoelectric printhead assembly [170, 171].

To eject a drop, an electric voltage is applied to deform the piezoelectric element. Application of the voltage across the actuator produces the mechanical stress in the piezoelectric element and causes the volume change in the chamber to generate a pressure wave that propagates toward the nozzle. The generated stress and mechanical properties can be found by equation 3.1.

$$\begin{aligned} V_{ef} &= mP; \\ \varepsilon &= \alpha V_{ef}; \\ E &= \frac{1}{m\alpha} \end{aligned} \quad (\text{Equation 3.1})$$

where V_{ef} is the applied electric field (V/m), P is the generated stress (Pa), m is a constant, ε is the induced strain, α is the piezoelectric coefficient, and E is the Young's modulus of the piezoelectric material. The acoustic energy wave and the volumetric constriction together tend to force the fluid in two directions, back towards the feed line and forward towards the orifice. Since the feed line is filled with non-compressible fluid, the path of least resistance is towards the orifice. Also, the fluid in the feed line acts as a barrier and acoustically reflects some portion of the acoustic

wave energy. Provided that the total energy level is sufficient to overcome the meniscus and resistive forces in the orifice, a small drop of material will be jetted.

Since the printhead relies on the constructive interference of pressure waves, the pulse width is the critical parameter for optimum droplet ejection. The effect of pulse width is discussed in the following section.

3.2.2 Pulse width effect

An electrical pulse applied to the piezoelectric printhead can be divided into three stages as shown in Figure 3.7 [171, 172].

Figure 3.7: Pulse width effect on inkjet printhead.

In phase I, upon powering the system, the jet tube is held at a voltage with the piezo tube poled. The ink chamber expands and initiates wave propagation in phase II. When the drop eject signal is received at phase III, the voltage drops and the piezo tube is depoled. Depoling causes the piezo tube to apply a constrictive force to the jet tube, reducing the chamber volume and sends an energy wave through the fluid residing in the jet tube [171] .

The tube is idealized as having open (supply end) and closed ends (orifice end) in Figure 3.8. The length of the tube is represented by l .

Figure 3.8: Idealized ink chamber.

According to Bogoy and Talkep, the optimum pulse width is l/c , where c is the acoustic velocity [172]. The optimum time frame between the applications of voltage step ejection of drop is $4l/c$. The conclusion is achieved based on a linear acoustic wave model [172].

3.2.3 Droplet formation

The acoustic wave interacts with the free meniscus surface at the nozzle to eject a droplet. Two important parameters that govern the droplet formation profile are the pressure and velocity response in the ink chamber. Researchers have used a characteristic method to solve the one-dimensional wave equation to obtain the transient pressure and velocity response [172, 173]. The droplet velocity at the nozzle exit plane will essentially determine the kinetic energy carried and hence its subsequent spreading behaviour. The solution is obtained by performing numerical calculation, which is beyond the scope of this study. It is the objective of this report to relate the operating parameter to the part resolution without looking at the detailed history inside the printhead. Droplet generated from a 3-D inkjet printhead is shown in a time-lapse picture in Figure 3.9 [170].

Figure 3.9: Droplet generated [170].

It can be seen that the droplets generated are spherical in shape with a consistent diameter and no satellite droplets are formed. The characteristic head-and-tail profile is shown clearly near the orifice of the printhead. The tail properties and break-up are mainly determined by the fluid properties such as surface tension and viscosity. Table 3.1 shows some quoted value for printing of build material at the following printing conditions [170].

Voltage amplitude	50V
Pulse width	30μs
Frequency	6kHz
Droplet size	~70μm
Droplet volume	500pL
Droplet velocity	3m/s

Table 3.1: Inkjet printing conditions.

From the table, one can conclude that the printing operation of T612 involves small droplets (micron size diameter) with high-speed impact (3m/s).

3.2.4 Droplet impact and spreading

Upon impact, a few scenarios can be derived from the droplet-substrate interaction. The droplet might spread, experience splashing or bounce off depending on the fluid properties and the droplet momentum (see Figure 3.10). The droplet bouncing off is usually observed in the deposition of high-density molten solder droplet [174].

Figure 3.10: Droplet impact.

During printing, the droplet momentum is controlled to avoid splashing. The droplet spreads upon impacting the substrate. Spreading of droplet is a function of droplet size, speed, liquid surface tension and substrate wetting properties. The spreading profile defines the lateral resolution of the system and the layer thickness.

In this section, the dimensions of a droplet on substrate are estimated as the maximum spread diameter and the droplet height. The formula for spread ratio is derived from a balance of energy (section (a)), while two models (section (b) and (c)) are proposed to estimate the droplet height.

(a) Balance of energy model

Referring to Figure 3.11, the amount of spreading is determined by the balance of driving and resisting forces.

Figure 3.11: Droplet impact and spreading.

The driving force for impact spreading is kinetic energy (KE) of the droplet, whereas flow resistance is provided by viscosity and surface energy (SE) of the liquid [175].

From the balance of energy:

$$KE_1 + SE_1 = KE_2 + SE_2 + W \quad (\text{Equation 3.2})$$

where subscripts 1 and 2 indicate before and after impact respectively and W is the work done to deform the droplet.

Defining the variables velocity as V , material density as ρ , viscosity as μ and surface tension as σ , Equation 3.2 is further detailed as follows:

Before impact, the droplet assumes a sphere mass with the diameter of d_o :

$$KE_1 = \frac{2\pi}{3} \rho V^2 d_o^3 ;$$

$$SE_1 = \pi d_o^2 \sigma$$

Upon impact, the droplet spreads to maximum diameter:

$$KE_2 = 0 ;$$

$$SE_2 = \frac{\pi}{4} d_m^2 \sigma (1 - \cos \theta)$$

The work done to deform the droplet against viscosity is

$$W = \frac{\pi}{3} \rho V^2 d_o d_m^2 \frac{1}{\sqrt{Re}}$$

Reynolds number, Re , indicates the relative importance of kinetic energy against viscous resistance for the spreading. The formula of Re is given by $\frac{\rho V d_o}{\mu}$. The relative magnitudes of the kinetic and surface energies can be expressed with Weber number, We , where $We = \frac{\rho V^2 d_o}{\sigma}$.

Hence, from equation 3.2, one can arrive at the maximum spread diameter as the spread ratio, ξ , in terms of We and Re as

$$\frac{d_m}{d_o} = \xi = \sqrt{\frac{We + 12}{3(1 - \cos \theta) + \frac{4We}{\sqrt{Re}}}} \quad (\text{Equation 3.3})$$

The contact angle, θ , is considered as the equilibrium solidification angle since the viscosity of the material impedes the recoiling motion of the droplet.

(b) Spherical cap model

The droplet height, h_{drop} can be derived from the conservation of droplet mass (see Figure 3.12).

Figure 3.12: Spherical cap on substrate.

The volume of the droplet (diameter d_o), Vol_{drop} , is equivalent to the volume of the spherical cap, Vol_{cap} , on the substrate after impact.

$$Vol_{drop} = \frac{\pi}{6} d_o^3; Vol_{cap} = \frac{\pi}{3} h_{drop}^2 (3R_{sphere} - h_{drop}) \quad (\text{Equation 3.4})$$

where R_{sphere} is the radius of the spherical cap.

Expressing the volume of the spherical cap in terms of θ ,

$$Vol_{cap} = \frac{\pi}{3} \left(\frac{d_m}{2 \sin \theta} \right)^3 (1 - \cos \theta)^2 (2 + \cos \theta) \quad (\text{Equation 3.5})$$

Rearranging equations 3.4 and 3.5, the spread ratio can be obtained as

$$\frac{d_m}{d_o} = \left[\frac{4 \sin^3 \theta}{(1 - \cos \theta)^2 (2 + \cos \theta)} \right]^{\frac{1}{3}} \quad (\text{Equation 3.6})$$

Since

$$h_{drop} = R_{sphere} - R_{sphere} \cos \theta \quad (\text{Equation 3.7})$$

where

$$R_{sphere} \sin \theta = r_b$$

Therefore the height of the droplet, h_{drop} , on substrate is calculated as:

$$h_{drop} = \frac{d_m}{2} \frac{(1 - \cos \theta)}{\sin \theta} \quad (\text{Equation 3.8})$$

Equation 3.8 shows that the height of the droplet is proportional to the solidification contact angle. A larger angle indicates less spreading which translates into a higher droplet height.

(c) Cylindrical model

A cylindrical model is suggested to simplify the estimation of the height of the droplet (see Figure 3.13):

Figure 3.13: Cylindrical model.

The droplet is modelled as a cylinder with base radius $d_m/2$ and height h_{drop} . By mass conservation, the volume of the cylinder is the same as that of the droplet, thus:

$$\frac{\pi}{4} d_m^2 h_{drop} = \frac{\pi}{6} d_o^3 \tag{Equation 3.9}$$

Simplifying, the height of droplet using a cylinder model is given as follows:

$$h_{drop} = \frac{2d_o^3}{3d_m^2} \tag{Equation 3.10}$$

Equation 3.10 shows that the droplet height is inversely proportional to the spread diameter.

In summary, there are three models available for the calculation of droplet dimensions deposited on the substrate. In the energy model, the balance of energy involving fluid properties is taken into account, which makes it a comprehensive model for the estimation of spread ratio. For the estimation of droplet height, accurate estimation can be obtained from the spherical cap model that follows the shape of a real droplet. The cylindrical approximation is a further simplified case and easier to model. Table 3.2 shows the values obtained from the models and these values are compared to the values quoted from the patent of the 3-D inkjet printer [171].

	(a)Energy model	(b)Spherical cap model	(c)Cylindrical model	Quoted value
Spread ratio	1.25	1.10	Not estimated	1.34
Droplet Height (μm)	Not estimated	23.6	28.4	30
Droplet diameter, $d_o = 76\mu\text{m}$, Spread diameter, $d_m = 102\mu\text{m}$ [171], Measured solidification angle, $\theta = 50^\circ$, $V = 3.0\text{m/s}$; $\sigma = 0.038 \text{ J/m}^2$; $\rho = 1150\text{kg/m}^3$; $\mu = 0.015 \text{ Pa.s}$.				

Table 3.2: Comparison of droplet models for InduraCast droplets.

The value of the spread ratio obtained using the energy model is very close to the quoted value, with a difference of 7%. The cylindrical model offers reasonable accuracy compared to the spherical cap model in estimating the height of a single droplet. The calculated droplet height is comparable to the quoted value at a deviation of approximately 5%. Therefore the cylindrical model is adopted in the subsequent modelling of layer thickness.

3.2.5 Successive deposition of adjacent droplet

In the construction of a 3-D part, the basic building block is a line of droplets instead of a single droplet. A line is formed when the deposited droplets are pushed in contact with each other and coalesce in the lateral direction due to capillary action (see Figure 3.14).

Figure 3.14: Successive deposition of adjacent droplet.

Hence, it is important to understand the dynamics of line formation in the printing process. The important parameter is the distance between the droplets, D . It is controlled by the scanning speed of the printhead, U , as well as the frequency of droplet ejection, f .

$$D = \frac{U}{f} \quad (\text{Equation 3.11})$$

Separated droplets will form if D is too large. Too small a value of D will cause formation of a bulging line since a slower printhead speed will deposit more droplets per unit length. The continuity of the line is governed by the following equation.

$$\frac{U}{f} < d_m \quad (\text{Equation 3.12})$$

Figure 3.15 illustrates the significance of the ratio U/f in Equation 3.12.

Figure 3.15: Line formation process as a function of frequency and print speed.

From equation 3.12, the upper limit of ratio U/f ensures the continuity of a printed line. A smaller value of ratio U/f represents a higher mass deposition rate or smaller distance between successive droplets. As a result, a line with higher layer thickness is formed.

The thickness of a line, h_{line} , can be derived by using the principle of mass conservation. From mass conservation, the material deposited on the substrate is equal to the material generated from the printhead. The cross sectional area of line is approximated as a rectangle as shown in Figure 3.16.

Figure 3.16: Modelling the height of a line.

The derivation for layer thickness as a function of frequency and velocity is shown as follows:

The volume of the line printed on the substrate is equivalent to the volume of droplets generated from the printhead.

$$A \cdot s = Vol_{drop} \cdot N_{drop} \quad (\text{Equation 3.13})$$

where A is the cross section of a line, s is the distance traveled by the printhead, Vol_{drop} as the volume of a droplet and N_{drop} is the number of droplets generated.

Replacing s and N_{drop} in equation 3.13,

$$A \times U \times t = Vol_{drop} \times f \times t \quad (\text{Equation 3.14})$$

where U is the scan velocity of printhead, f is the frequency of droplet ejection and t is the time taken to form the line.

Adopting the cylindrical model in section 3.2.5, the cross sectional area of the line is approximated as a rectangular shape with w and h_{line} as the width and height of line respectively.

$$A \approx w \times h_{line} \quad (\text{Equation 3.15})$$

Hence, the height of the line is defined as:

$$h_{line} = \frac{Vol_{drop}}{w} \cdot \frac{f}{U} \quad (\text{Equation 3.16})$$

Substituting $w = d_m = \xi d_o$ into equation 3.16, the height of a line can be expressed as a function of frequency and print speed as shown below.

$$h_{line} = \frac{Vol_{drop}}{\xi d_o} \cdot \frac{f}{U} \quad (\text{Equation 3.17})$$

Substituting equation 3.3 into equation 3.17, h_{line} is expressed as a function of droplet volume, diameter, ejected velocity, contact angle, frequency, viscosity and surface tension of the material.

$$h_{line} = \frac{Vol_{drop}}{d_o} \cdot \frac{f}{U} \left[\frac{We + 12}{3(1 - \cos \theta) + \frac{4We}{\sqrt{Re}}} \right]^{\frac{1}{2}} \quad (\text{Equation 3.18})$$

Equation 3.18 shows that the layer thickness is affected by the ratio of frequency over printhead velocity. The thickness increases as printing frequency increases, or when the printhead moves at a slower speed. Higher frequency translates into higher mass

transfer rate while a slower speed allows more material to be deposited at the particular spot.

h_{line} is also an inverse function of the spread ratio. The width of the line is not significantly affected by the change of the frequency. This is mainly due to the rapid equilibrium achieved by moving the contact line. Rapid solidification of droplets limits the lateral deformation of the line. Hence, the spread ratio is considered as a constant value in equation 3.17. Table 3.3 shows a sample calculation for the droplet height with the respective printing condition using equation 3.18.

Volume of a droplet	500pL
Width	101.6μm
Frequency	6kHz
Velocity of printhead	0.18m/s
Height	164μm

Table 3.3: Sample calculation for drop height.

However, the material deposition rate cannot be too high such that the internal fluid pressure in the line exceeds the surface tension that maintains the parallel sides of the line.

3.2.6 Successive deposition of layer

In the T612, the deposited layer thickness is allowed to solidify and is then milled by the cutter to achieve the specified layer thickness (see Figure 3.17). Subsequently, the next layer is deposited on a substrate of the same material.

Figure 3.17: Successive deposition of layers.

In the 3D printing process, the droplet is assumed to impact on the substrate at the operating temperature of 120 °C for the build material, InduraCast; and 110 °C for the support material, InduraSupport. These values are considerably higher than the

melting temperature of the individual material, which is 106°C and 72 °C respectively. Hence, the substrate of identical material may initially melt due to the energy transfer from the impacting droplet, a phenomenon sometimes called remelting in the literature [176].

Understanding the remelting phenomenon is important for the optimization of bonding strength between layers. In the case of insufficient remelting, the quality of the material can suffer from poor adhesion between the droplets; on the other hand, if there is too much remelting, the resulting shape of the material can be inaccurate.

Layer thickness also affects the surface roughness of the printed part. The droplet-based process results in the edge of the layer having a rounded profile (see Figure 3.18). The droplets spread and flatten into a half spherical cap upon impact on the substrate. Hence, the edge defined by the material has a convex and rounded profile.

Figure 3.18: Rounded edge profile.

The expected surface roughness is the peak-to-peak value of the thickness of the deposited value. Figure 3.19(a) shows the closed-up view of the edge profile. A model is presented to measure the surface roughness as a function of layer thickness (see Figure 3.19(b)).

Figure 3.19: (a) SEM picture of edge profile after milling.
(b) Modelling the edge profile.

The peak-to-peak value, d_p can be related to the layer thickness, h by using simple trigonometry as shown in equation 3.19 (see Figure 3.20).

Figure 3.20: Modelling the peak-to-peak distance.

$$\tan \theta = \frac{h_{line}}{d_p} \quad (\text{Equation 3.19})$$

From definition of surface roughness, $R_a = \frac{I}{h_{line}}$ [177] where I is the edge area.

Since $I = \frac{1}{2} \times h_{line} \times d_p$, hence,

$$R_a = \frac{1}{2} d_p \quad (\text{Equation 3.20})$$

Substituting $d_p = \frac{h_{line}}{\tan \theta}$ and equation 3.17 into equation 3.20, the surface roughness is expressed as:

$$R_a = \frac{Vol_{drop}}{2\xi d_o \tan \theta U} f \quad (\text{Equation 3.21})$$

The surface roughness is expected to increase as the material deposition rate increases, resulting in a bigger peak-to-peak value.

3.3 Material Interaction

In the T612 deposition process, each layer comprises two materials. A first portion represents a cross-sectional slice of a three-dimensional object being built and is composed of the build material, InduraCast. The second portion (InduraSupport) is the complement of the object shape of the first material and serves as a support structure that supports the growing object. This enables the construction of intricate shapes and parts having undercut features.

3.3.1 Roundness of circular profile

A small slab (10mm×5mm) of 1mm thickness with 3 through holes of decreasing diameter of 2.0mm, 1.0mm, and 0.55mm respectively, is printed to demonstrate the importance of build-support material interaction (see Figure 3.21). The through hole is originally occupied by support material during the printing process to maintain a round profile through the slab. The support material has been removed in Figure 3.21(a).

Figure 3.21: Round profile printed.

The roundness of the profiles in Figure 3.21(b)-(c) was analyzed using Minimum Radial Separation (MRS) method according to the ANSI Y14.5M-1982 standard [178, 179]. In this method, two concentric circles with minimum radial separation were chosen to contain all the data points of the profile (see Figure 3.22). The radial

difference between the concentric circles determined by this method is the measure of the out-of-roundness value (OOR). The concentric circles were obtained by tracing an inscribed circle using two inner contact points followed by tracing a concentric circumscribing circle using an outer contact point.

Figure 3.22: Minimum radial separation method.

The circumferential profile of the through holes in Figure 3.21 (b)-(d) was outlined using a public domain Java image-processing program, ImageJ. The profile was then superimposed on a polar plot to determine the location and size of the two reference circles (see Figure 3.23). The OOR value was measured using ImageJ.

Figure 3.23: Circular profile on a polar plot.

Figure 3.24 shows the application of MRS method on the circular profiles obtained. The out-of-roundness values were indicated on the figures.

Figure 3.24: Out-of-roundness of the circle profiles.

Microscopic picture taken (see Figure 3.24) shows that the through holes have a jagged edge at the perimeter. The measured OOR values were 188μm, 127μm and 108μm respectively. This shows that the radial deviation from the intended round

profile was larger at a larger diameter of the through hole. Such undesirable feature might be due to the inadequate support for build material during printing, causing excessive spreading of the build material.

3.3.2 Overhang feature

The significance of support-build material interaction is even more prominent in the printing of overhanging feature since the overhanging feature is essentially printed on top of the support material. Failure to deposit the support material accurately will impair the precision of the final part profile.

Figure 3.25 shows one example of such feature while the plan view of the feature is shown in Figure 3.26.

Figure 3.25: Side view of a part.

Figure 3.26: Plan view of the overhanging feature.

Figure 3.25 is a side view of a part. The design utilizes a column as a support for the overhanging feature. The void is a space left after removing the support material. Microscopic picture of the overhanging feature shows that the corner is not square and the edges of the lines are not straight (see Figure 3.26). Unsatisfactory support for the overhang feature is the main reason for such imprecision.

Build material is often applied at a sufficient temperature relative to the melting point of the support material so as to melt the support structure. Therefore, the support material should be able to withstand contact with the build material without melting.

3.4 Summary

This chapter discusses the theory behind the 3-D inkjet printing with some experimental work. The Solidscape T612 operates a droplet-based process in which the smallest building block of a part printed is the droplet. The droplet generated also defines the minimum resolution of this process. These droplets need to have a consistent volume and ejection speed as these two parameters determine momentum of the droplet. Consequently, the momentum of the droplet would determine the extent of spreading upon impact.

As discussed in Sections 3.2.1 to 3.2.3, important parameters that determine the momentum of the droplets are the input signal (pulse width, voltage amplitude) and the piezoelectric response of the printhead. Theoretical development was presented to illustrate the phenomena of liquid droplet formation, ejection, and impact on the substrate. With the understanding of these phenomena, it is then possible to predict and control the profile of a deposited droplet using the models presented in Section 3.2.4.

The layer thickness is an important performance index for rapid prototyping since it is a layer-by-layer manufacturing process. A model was presented in Section 3.2.5 to relate the process parameters (printhead frequency and velocity) to the layer thickness. Surface roughness of the part produced was found to be dependent on the layer thickness as well.

Subsequently, the importance of support-build material interaction was illustrated with experimental work in Section 3.3. It was shown that the build-support material interaction governs the accuracy of the printed part geometry.

CHAPTER 4

SCAFFOLD DESIGN

Current research in the fabrication of TE scaffold has seen limited advancement in the design of a scaffold especially in the aspect of theoretical derivations. In this study, flow channels were incorporated into the matrix as part of the pre-determined architecture for homogenous perfusion of culture medium over the scaffold. Theoretical derivations, which define the channel morphology, were developed based on the physiochemistry requirement of the scaffold. The internal channel mould design was generated using a computer-aided-design system. An analytical model is also described at the end of this chapter to illustrate the enhancement of cell proliferation in scaffold with the internal channels.

4.1 Internal Channel Design

As discussed in Chapter 2, it is important to seed cells into a scaffold with sufficient cell density and in a homogenous fashion. The cell seeding method is coupled to a bioreactor or flow systems where dissolved oxygen is provided by the circulating culture medium (see Figure 4.1). Hence, the hypothesis is that the perfusion of the culture medium through the scaffold would be more efficient with the incorporation of interconnected flow channels.

Figure 4.1: Flow channel.

In this hypothesis, the most important parameter is the volumetric flow rate, which is a function of the flow channel dimension. The optimal channel dimension is estimated by considering two models, namely the estimated flow shear and oxygen mass balance in the scaffold. A section of the scaffold with flow channel was selected as

the control volume for the analysis. An analytical model is also described at the end of this chapter to illustrate the enhancement of cell proliferation in scaffold with the internal channels.

4.1.1 Estimated flow shear

The flow channel is considered to assume a circular cross section with a diameter, d_{ch} (see Figure 4.2).

Figure 4.2: Flow channel delivering culture media.

Consider a specific volume of flow carrying oxygen and rich in nutrient is delivered through the channel at flow rate, Q . The average velocity of the flow, V , is equal to the flow rate divided by the cross sectional area of the channel.

$$V = \frac{Q}{\pi \left(\frac{d_{ch}}{2} \right)^2} \quad (\text{Equation 4.1})$$

Fluid shear stress, τ , for a circular channel is assumed to be of a flow through pores, which can be described by equation 4.2.

$$\tau = \frac{8\mu V}{d_{ch}} \quad (\text{Equation 4.2})$$

where μ is viscosity of the culture medium.

Hence the flow rate as a function of channel cross-section area is:

$$Q = \frac{\pi d_{ch}^3 \tau}{32\mu} \quad (\text{Equation 4.3})$$

If the shear stress that a particular cell type “prefers” can be determined, the desired volumetric flow rate, Q can then be estimated.

4.1.2 Oxygen mass balance in element

The flow rate, Q , necessary for a given tissue culture can be estimated from a simple mass balance, with the simplifying assumption that each cell metabolizes oxygen at a constant rate, R_{O_2} . The amount of oxygen entering the control volume is given by the volumetric flow rate multiplied by the concentration of oxygen. The difference between the inflow concentration and outflow concentration is equal to the amount of metabolized oxygen, given by the rate of oxygen uptake per cell multiplied by the estimated number of cells in the control volume, N_{cell} .

Assuming that the whole control volume of dimensions length, L , width, b_{sc} , and height, h_{sc} , with internal channel of diameter, d_{ch} , is occupied by cells (see Figure 4.3).

Figure 4.3: Model of scaffold control volume.

The net volume available for cells occupation is Vol_{net} :

$$Vol_{net} = [b_{sc} h_{sc} L - \pi (\frac{d_{ch}}{2})^2 L] \phi \quad (\text{Equation 4.4})$$

where ϕ is the void fraction or porosity.

Hence, the maximum number of cells, N_{cell} , of diameter, d_{cell} , and volume, Vol_{cell} in a scaffold, is

$$N_{cell} = \frac{Vol_{net}}{Vol_{cell}} \quad (\text{Equation 4.5})$$

The amount of oxygen dissipating through the scaffold is the amount of oxygen consumption rate, R_{O_2} , multiplied by the number of cells. It is equivalent to the

volumetric flow rate multiplied by the difference in concentration of oxygen at the inlet, C_{in} , and outlet, C_{out} .

$$Q(C_{in} - C_{out}) = N_{cell} R_{O_2} \quad (\text{Equation 4.6})$$

Rearranging and substituting the difference in oxygen concentration as a ratio,

$$Q = \frac{N_{cell} R_{O_2}}{\psi C_{O_2}} \quad (\text{Equation 4.7})$$

where ψ is a ratio of concentration defined as:

$$\psi = \frac{C_{in} - C_{out}}{C_{in}} \quad (\text{Equation 4.8})$$

Combining equations 4.4 and 4.7 and assuming a rectangular scaffold with dimensions of length, L , width, b_{sc} and height h_{sc} , the length of channel, L , is

$$\therefore L = Q \frac{\psi C_{O_2}}{R_{O_2}} \frac{Vol_{cell}}{\phi [b_{sc} h_{sc} - \pi (\frac{d_{ch}}{2})^2]} \quad (\text{Equation 4.9})$$

Oxygen concentration, C_{O_2} , can be estimated from thermodynamic saturation values, assuming equilibrium with an atmosphere of 21% O_2 at 37°C and 1 atm. Consumption rate data and typical cell diameter values can be obtained from experiments. The fraction of oxygen used, ψ is the minimum allowable oxygen level in the scaffold.

4.1.3 Estimated channel length

The final dimension of the control volume can be estimated using equation 4.9 and the values cited in Table 4.1.

$$\therefore L = Q \frac{\psi C_{O_2}}{R_{O_2}} \frac{Vol_{cell}}{\phi [b_{sc} h_{sc} - \pi (\frac{d_{ch}}{2})^2]} = 8.6 \text{ mm}$$

This calculated value serves as the maximum value of the channel length limited by amount of oxygen delivered by the specific flow rate.

Parameters	Symbol	Value cited
<u>Cellular parameters</u>		
Cell diameter	d_{cell}	20 μm [180]
Cell volume (sphere)	Vol_{cell}	$3.14 \times 10^{-9} \text{cm}^3$
<u>Physiological parameters</u>		
Per-cell oxygen consumption rate	R_{O_2}	$8.3 \times 10^{-16} \text{mol/cell-s}$ [181]
Minimum allowable oxygen level	ψ	0.5
Shear stress on cell membrane	τ	1.0 Pa [182]
<u>Fluid parameters</u>		
Concentration of oxygen in culture medium at 37°C	C_{O_2}	$1.5 \times 10^{-7} \text{mol/cm}^3$ [134]
Viscosity of culture medium	μ	0.001 Pa-s [143]
<u>Scaffold parameters</u>		
Diameter of channel	d_{ch}	500 μm
Porosity	ϕ	0.5
Width	b_{sc}	900 μm
Height	h_{sc}	900 μm

Table 4.1: Relevant parameters used.

4.2 Internal Channels Design using CASTS

The mould was generated using a system established in-house that could be used to design scaffold internal architectures from a selection of open-cell polyhedral shapes.

4.2.1 CASTS system

The prototype system, called the computer-aided system for tissue scaffolds (CASTS), has a parametric library of design units which can be assembled into scaffold structures through a stitching algorithm [183]. The automated scaffold assembly algorithm can be interfaced with various RP technologies to achieve automated production of scaffolds.

In this system, a total of 13 configurations were created using different polyhedral shapes as referencing basis (see Figure 4.4).

Figure 4.4: Examples of basic polyhedral shapes of CASTS and the assembled structures [184].

The system was developed on the platform of a commercial available design software, Pro-Engineer. A total of 80 parameters are involved in the system including user input parameters and dependent parameters. Through this system it is possible to control the density, diameter, configuration and the orientation of the channels.

Some examples of defining parameters of the systems are shown in Table 4.2.

User input parameters

- Polyhedral packing configuration
- Name of cellular unit
- Presence of node
- Overall unit cell length
- Overall unit cell breadth
- Overall unit cell height
- Strut diameter
- Overall scaffold size

Dependent parameters

- Surface to volume ratio
 - Porosity
 - Maximum pore size
 - Minimum pore size
 - Scaffold length resolution
 - Scaffold breadth resolution
 - Scaffold height resolution
 - Cellular unit quantity
-

Table 4.2: Parameters of CASTS.

4.2.2 Selected configuration

The selection and sizing of the basic polyhedral shape was based on the criteria of: (1) ease of software generation, (2) ease of cleaning, (3) sufficient handling strength of the printed mould, (4) sufficient channels interconnectivity and (5) ease of casting at later stage.

Configuration 5, the Square Pyramid, was selected based on these criteria (see Figure 4.5). The basic polyhedral shape is a square pyramid with 4 diagonally positioned internal struts that intercept at the center of the square. The struts are 500 μ m in diameter and the overall size of a square is 4mm $\times$ 4mm $\times$ 4mm.

Figure 4.5: Basic polyhedral shape of selected configuration.

4.2.3 Internal channels mould design

The scaffold and consequently the mould, was specified to have an enveloping cylindrical shape with 15mm diameter and 4mm thickness. The overall dimension of the scaffold was chosen to provide enough test volume for the investigation of the internal architecture while maintaining high efficiency of production. The mould design is shown in Figure 4.6.

Figure 4.6: Internal channel mould design using CASTS.

The mould was generated using the square pyramid as the basic unit cell. It served as the proof-of-concept design for the investigation of indirect fabrication technique in this project. The processing steps developed would be applicable to other design of configurations as well.

4.3 Enhancement of Cell Proliferation with Internal Channels

One major challenge in scaffold-based tissue engineering has been the limitation of cell migration and tissue ingrowth within the scaffolds. The channels dispersing through the entire scaffold volume were designed to serve as flow conduits that help to deliver fresh medium throughout the entire scaffold. Hence, it is possible to maintain the viability of cell even at the central locations. Therefore it is believed that the internal channels incorporated could enhance the cell proliferation in the collagen scaffold. An analytical model is presented to illustrate the enhancement quantitatively.

4.3.1 Maximum number of cells in scaffold

The maximum number of cells sustained in a scaffold limited by nutrient diffusion can be derived using Fick's law and a simplified one-dimensional (1-D) model. Fick's law describes the fundamental relationship between diffusive flux and solute concentration. This law was used to develop the general mass conservation equations of a control volume. The model is presented in Figure 4.7.

Figure 4.7: Simplified 1-D diffusion model.

The balance equation of oxygen can be written as equation 4.10 assuming that oxygen is consumed without accumulation in the scaffold.

$$J_x A_{sc} - J_{x+\Delta x} A_{sc} - \hat{R}_{O_2} A_{sc} \Delta x = 0 \quad (\text{Equation 4.10})$$

where J is the diffusion flux, A_{sc} represents the area, $\hat{R}_{O_2} = R_{O_2} \hat{N}$ and $\hat{N} = \frac{\text{cell}}{\text{Volume}}$

$$\text{Hence, } \frac{\partial J}{\partial x} = -\hat{R}_{O_2} \quad (\text{Equation 4.11})$$

According to Fick's Law, the concentration gradient of oxygen in the scaffold is directly related to the oxygen flux through the scaffold.

$$J = -D_{O_2} \frac{dC_{O_2}}{dx} \quad (\text{Equation 4.12})$$

where D_{O_2} is the diffusivity of oxygen in culture.

Differentiating equation 4.12 and combining equation 4.11

$$D_{O_2} \frac{d^2 C_{O_2}}{dx^2} = \hat{R}_{O_2} = R_{O_2} \hat{N} \quad (\text{Equation 4.13})$$

The general solution for equation 4.13 is

$$C_{O_2} = \frac{R_{O_2} \hat{N}}{D_{O_2}} \frac{x^2}{2} + A_1 x + A_2 \quad (\text{Equation 4.14})$$

Specific solution can be obtained by applying the following boundary conditions:

(1) At $x = 0$, $\frac{dC_{O_2}}{dx} = 0$ where there's no gradient and diffusion at the base plane.

$$\therefore A_1 = 0$$

(2) At $x = h$, $C_{O_2} = C_0$ where the concentration of oxygen is the same in the bulk

culture medium. $\therefore A_2 = C_0 - \frac{R_{O_2} \hat{N} h^2}{2D_{O_2}}$

Hence, the solution for the concentration of oxygen C_{O_2} in the scaffold is:

$$C_{O_2} = C_0 - \frac{R_{O_2} \hat{N}}{2D_{O_2}} (h^2 - x^2) \quad (\text{Equation 4.15})$$

Using $\psi = 0.5$ from the previous calculation and substituting $C_{O_2}(x=0) = 0.5C_0$ into equation 4.15, equation 4.16 was obtained.

$$\frac{1}{2} = \frac{R_{O_2} \hat{N}}{2D_{O_2} C_0} h^2 \quad (\text{Equation 4.16})$$

Rearranging the equation to obtain the equation for number of cells in scaffold.

$$\hat{N} = \frac{C_0 D_{O_2}}{R_{O_2} h^2} \quad (\text{Equation 4.17})$$

A closer examination on equation 4.17 showed that it is the limiting case of Thiele ratio, S . Thiele ratio is a dimensionless ratio of consumption to supply [185]. The definition of Thiele ratio is shown in equation 4.18.

$$S = \frac{R_{O_2} \hat{N}_x^2}{D_{O_2} C_{O_2}} \quad (\text{Equation 4.18})$$

The demand is greater than the supply when $S \gg 1$. At $S \ll 1$, the supply exceeds demand. The value of S was assumed to be 1.0, indicating that the supply is balanced by the demand of oxygen in the scaffold [134].

Applying the 1-D model to the RP scaffold with interconnected internal channels, the concentration of oxygen in the channels is equal to the surrounding medium as the medium from the well can be delivered into the scaffold via these channels. Hence parameter h in the case of RP scaffold is the thickness between two channels. The channels are well dispersed across the thickness of the scaffold in 3-D manners. Hence, $h = 2\text{mm}$ was selected as the average value for this parameter.

Quantitative analysis is presented in the following Table:

	Scaffold without internal channels	Scaffold with internal channels
Thickness, h	4mm	2mm
Number of cells, $\hat{N}$	1.48×10^4 cells/volume	5.92×10^4 cells/volume
Concentration of oxygen in medium, $C_{O_2} = 1.5 \times 10^{-7} \text{ mol/cm}^3$ [134]		
Diffusion coefficient of oxygen in water, $D_{O_2} = 1 \times 10^{-5} \text{ cm}^2/\text{s}$ [186]		
Per-cell oxygen consumption rate, $R_{O_2} = 8.3 \times 10^{-16} \text{ mol/cell-s}$ [181]		

Table 4.3: Maximum number of cells sustained in scaffold.

From the analytical modelling and the sample calculation, it can be observed that the incorporation of internal channels would significantly enhance the proliferation of cell in scaffold compared to the ones without internal channels.

4.4 Summary

The indirect rapid prototyping method offers great flexibility in the design of the scaffold. Biomaterial can be shaped as desired according to the design of the sacrificial mould. This chapter presents the analysis and calculations for the design of the mould. In this design, interconnected channels are incorporated to act as flow channels that would increase the mass diffusion efficiency through the scaffold. The calculations of the channels lengths have been performed based on the estimated flow shear in the channels and the oxygen mass balance required in the scaffold.

The part printed should have sufficient handling strength and is able to withstand the cleaning process without breaking. Another important design consideration of the mould is the ease of flowing of the cast material. A final design with branching channels is generated using an in-house developed automated algorithm. An analytical model is presented at the end of this chapter to illustrate the enhancement of cell proliferation in scaffold with the internal channels.

CHAPTER 5

SCAFFOLD REALIZATION AND CHARACTERIZATION

5.1 Collagen Scaffold Realization

Collagen has been used to prepare a variety of medical products such as corneal shields, wound dressing and catheter cuffs due to its intrinsic biological and physiochemical characteristics [43]. Collagen scaffolds are also widely used in tissue engineering [18, 43, 44, 55, 56, 70, 187, 188].

These collagen scaffolds were normally prepared by a freeze-drying process and reported as porous cylindrical sponge. However, the freeze-drying process offers limited control on the design of the scaffold. The internal pores structure of freeze-dried collagen scaffold is random and are usually not fully interconnected. It is a challenge to control the morphology of collagen scaffold as collagen is thermally sensitive and denatures at 40°C [189].

As a result, indirect RP processing technique was developed in this research in order to overcome the challenges in shaping collagen and to realize the design of porous collagen scaffold with 3-D internal channels. The steps are detailed in the following sections. A comparative study was performed on two different drying techniques, namely the critical point drying and the freeze-drying method. Various types of analysis on the characterization of the scaffold are also outlined. The characterizations were performed on (a) the mould, (b) the scaffold, and (c) the cell-seeded scaffold. The cell culture protocol and the characterization techniques for *in vitro* evaluation of the collagen scaffolds are presented. Two different culturing techniques namely the static and the direct perfusion culture are described.

5.2 Indirect RP Method

The proposed indirect RP method process flow is presented in Figure 5.1 and each step will be discussed in detail in the following sections.

Figure 5.1: Process flow of indirect RP method.

5.2.1 Design of mould

The design of the mould was carried out using Pro-Engineer software. The CAD models can be exported in STL (StereoLithography) file. The STL format has been used as the *de facto* standard in all RP systems [76]. Subsequent data handling was carried out using the manufacturer's application software, ModelWorks™. The CAD file was orientated along the printing axis (see Figure 5.2). ModelWorks™ allows the user to choose the build setting from a library of configurations. The settings for various parameters such as cutter feed rate, cooling profile and jet velocity were

predetermined in each configuration. The layer thickness specified was 0.0508mm for the fabrication of moulds.

Figure 5.2: ModelWorks™ interface.

5.2.2 Fabrication of mould

The mould was fabricated using 3D phase change inkjet printer T612 from Solidscape. The materials used for the mould were proprietary materials of Solidscape, InduraCast and the support material InduraFill [190]. The properties of these materials are presented in Table 5.1. The technical specifications of T612 are listed in Appendix A [190].

	InduraCast	InduraFill
Composition	Thermoplastic	Natural and synthetic waxes and fatty esters
Melting point	95-110 °C	49-70 °C
Density	1250 kg/m ³	810 kg/m ³
Viscosity	15 mPa.s	9 mPa.s
Surface tension	0.038 J/m ²	0.027 J/m ²

Table 5.1: Material properties.

After completion of the printing process, the parts were removed from the machine. The metal plate, which was the printing platform, was placed on a heater to break the cohesive bonding layer between the metal plate and the substrate. The parts were then immersed in a mineral oil bath at 60° - 65° C to remove the support material. Figure 5.3 shows the 3-D printed mould according to the CAD design.

Figure 5.3: Fabrication of mould.

5.2.3 Casting of material

Collagen Type I (Bovine Achilles tendon from Sigma-Aldrich) was used as the scaffold material. Collagen scaffold material (1% weight/volume) was dispersed in 0.05M acetic acid and stirred for 5 hours at room temperature. The solution was degassed in vacuum to remove all air bubbles.

5.2.4 Freezing

Freezing the dissolution resulted in the production of ice crystals that grew and forced the collagen into the interstitial spaces, thus aggregating the collagen. The kinetics of the freezing mechanism was governed by heat transfer in the solution [191].

5.2.5 Removal of mould

The mould was removed by immersing the frozen collagen-mould construct into a bath of ethanol. Figure 5.4 shows the frozen construct in ethanol bath.

Figure 5.4: Frozen collagen-mould construct.

5.2.6 Drying of scaffold

The scaffold was dried to bring the scaffold to solid state at ambient pressure. In this research, two different drying methods namely, critical point drying and freeze-drying were experimented to produce a scaffold with less shrinkage, increased open porosity and interconnected 3-D channels as designed. The process flow is summarized in Figure 5.5. The resultant scaffolds were evaluated in terms of dimensional accuracy and internal morphology. The results are presented in Chapter 7.

Figure 5.5: Process flows under investigation.

5.2.6.1 Critical point drying technique

Critical point drying was carried out using a critical point dryer (CPD030, Bal-Tec, Germany). Briefly, the scaffolds were placed inside the ethanol-filled pressure chamber. The chamber was cooled to 10°C before the liquid CO₂ was added. The stirrer was turned on to aid the mixing of ethanol and liquid CO₂. Slowly, a few ml of liquid was dispensed from the chamber and a similar amount of liquid CO₂ was added. A total of 5 replacement cycles were carried out with 10 minutes' holding time to ensure proper mixing and replacement of ethanol with liquid CO₂. The chamber was then heated up to 10°C past the critical point of CO₂, which is at 31°C and 1072 psi, in order to prevent recondensation of the CO₂ liquid. A pressure relief valve was employed to slowly vent off excessive pressure. The scaffolds were then immersed in CO₂ gas without being exposed to the damaging surface tension forces. The overall process is summarized in Figure 5.6.

Figure 5.6: Critical point drying process.

5.2.6.2 Freeze-drying technique

After the ethanol treatment, the scaffolds were immersed in a large bath of distilled water and mechanically agitated for 5 hours to ensure the ethanol was completely replaced by water. The scaffolds were then frozen at -20°C for at least 8 hours and dried using a freeze dryer (Christ, alpha 1-2, Germany) for 24 hours with the ice condenser temperature at -55°C.

Freeze-drying involves the removal of water from the frozen scaffold by a sublimation process. Sublimation occurs when a frozen liquid goes directly to the gaseous state without passing through the liquid phase. The rate of sublimation of ice from the frozen scaffold depends upon the difference in vapour pressure of the scaffold compared to the vapour pressure of the ice collector. The frozen scaffold absorbs heat, causing water in the scaffold to enter the vapour phase and migrate into the instrument atmosphere where it is removed by refreezing on the condenser.

5.3 Characterization of RP Fabricated Mould

The resolution of the printed part would directly affect the resolution of the final scaffold since the morphology of the scaffold will take on the geometry of the mould. Therefore, the resolution of the machine was investigated to evaluate the performance of this process. The results are presented in Section 7.2.

5.3.1 Resolution of fabricated mould

A plane mould with overall dimensions of $11 \times 11 \times 3 \text{ mm}^3$ was printed. The branching internal channels were designed to have the widths of $600 \mu\text{m}$, $400 \mu\text{m}$ and $200 \mu\text{m}$ respectively (see Figure 5.7). The width of the channels was measured from micrographs acquired with a scanning electron microscope (SEM) (JSM-35CF, JEOL, Japan).

Figure 5.7: Branching channel.

5.3.2 Accuracy of internal channels

The mean diameter of the interconnected channels was determined by visual analysis of the SEM images. The moulds were sputter-coated with gold–palladium and images were taken using SEM (JSM-35CF, JEOL, Japan). Four channels were measured per image. The diameter was measured four times at different axis. A total of 6 images were analyzed. Then the average value was calculated and taken as the mean channel size.

5.4 Characterization of Collagen Scaffold

5.4.1 Evaluation of dimensional accuracy

The diameter and the thickness of three scaffolds were measured using a digital vernier caliper (Mitutoyo, USA). Three entries were made for each measurement at different locations and the average value was recorded. The linear shrinkage was calculated using equation 5.1.

$$\%shrinkage = \frac{L_0 - L_1}{L_0} \times 100\% \quad (\text{Equation 5.1})$$

Where L_0 and L_1 are the original dimension and the final dimension of the scaffold respectively.

5.4.2 Morphology microscopy analysis

Two scaffolds were cut diametrically to obtain a cross sectional area so as to expose the internal morphology (see Figure 5.8). The samples were then sputter-coated with gold–palladium and images were taken using an SEM machine (JSM-35CF, JEOL, Japan). The mean pore size of the scaffolds was determined by analysing of the SEM images. Twelve apparent pores were measured per image. The pores were measured in a perpendicular direction through the long axis and the minor axis. The average value was then calculated and taken as the mean pore size.

Figure 5.8: Morphology microscopy analysis.

5.4.3 Porosity

The porosity of the compliant collagen scaffolds was measured using two different techniques, namely the liquid displacement technique and the gas displacement technique. The first method was reported in many articles [192-194]. The scaffolds were immersed in a cylinder containing known volume of ethanol, V_1 , for 5 minutes, the total volume was recorded as V_2 . The ethanol-impregnated scaffold was removed from the ethanol and the remaining volume of ethanol was recorded as V_3 . The open porosity of the scaffold, ϕ_{liq} , is obtained from equation 5.2.

$$\phi_{liq} = \frac{(V_1 - V_3)}{(V_2 - V_3)} \quad (\text{Equation 5.2})$$

Alternatively, a gas displacement technique was investigated using a gas pycnometer (AccuPyc 1330 Pycnometer, Micromeritics, USA). The result of the solid phase volume, V_{solid} , was computed by the device from observing the pressures upon filling the sample chamber with helium gas and then discharging it into a second empty chamber. 20 purges were performed with equilibration rate of 0.0050 psig/min. With the overall dimension of the scaffolds known, the information of solid phase volume could be adapted to calculate the porosity of the scaffold using equation 5.3.

$$\phi_{gas} = 1 - \frac{V_{solid}}{V_{overall}} \quad (\text{Equation 5.3})$$

The overall volume of the scaffolds was calculated using the equation $V_{overall} = \pi r^2 h$, where r is the radius of the scaffold and h is the height. The mean height and diameter of the scaffolds were measured using a digital vernier caliper (Mitutoyo, USA).

5.4.4 Fourier transform infrared spectroscopy

A small amount of collagen scaffold was placed in potassium bromide crystals and hydraulic pressed into thin pellet. The infrared spectrum was obtained using a Fourier Transform Infra Red (FTIR) spectrometer (Spectrum GX, Perkin-Elmer, USA) in transmission between 370 and 4000 cm^{-1} with a resolution of 4 cm^{-1} .

5.4.5 Differential scanning calorimetry analysis

Thermal properties such as glass transition temperature and melting point of the collagen were determined by differential scanning calorimetry (DSC) using thermal analysis instruments (DSC 7, Perkin Elmer, USA).

5.5 *In Vitro* Evaluation of Collagen Scaffold

The *in vitro* performance of collagen scaffolds with 3-D channels fabricated by indirect RP technique (RP scaffold) was compared with conventionally freeze-dried collagen scaffold (control scaffold). Two different culturing techniques namely the static culture and the direct perfusion culture were investigated. Details are presented in the following sections.

5.5.1 Characteristics of SaOS-2 cells

In vitro performance of the collagen scaffolds was evaluated using immortalized Human Primary Osteogenic Sarcoma cells (SaOS-2). Immortalized cell lines are common alternative to primary human cell cultures in biomedical research considering the advantages that it can offer, which includes: (1) The cell line is easier to procure and maintain, (2) It is better characterized, and (3) The cell lines are less prone to phenotypic drift than their primary cell counterparts [195].

Osteosarcoma cell line SaOS-2 has been recognized as osteoblast-like and had been used in a number of studies to model behaviours of cells such as attachment, spreading, proliferation, and differentiation [195-198]. These cells can also be induced to differentiate along the osteoblastic lineage in culture, and, following differentiation; the cells can produce a mineralizing matrix *in vitro*. The morphology of SaOS-2 cells is epithelial-like (see Figure 5.9).

Figure 5.9: Epithelial-like SaOS-2 cells.

5.5.2 Culture and passaging protocol of SaOS-2 cells

SaOS-2 cells were obtained from The European Collection of Cell Cultures [199]. General information of the cell line is listed in Appendix B [199]. Cells were seeded onto a tissue culture flask in Minimum Essential Medium (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen) and 100 units/ml Penicillin-Streptomycin (Invitrogen). Cells were maintained at 37 °C in 5% CO₂-humified atmosphere and harvested physically using a rubber spatula by scrapping the cell from the attached surface when a monolayer was reached (see Figure 5.10).

Figure 5.10: Cells in flask.

Cells were routinely checked for common contamination such as bacterial, fungal and mycoplasma contamination. The culture medium in the flask was inspected to ensure no precipitates or shift in pH that could be caused by bacterial, yeast, or fungal infection. Cell growth pattern of the cells was monitored to ensure no obvious decrease in growth rate. Decreased growth of culture could be due to improper storage of reagents, low-level bacterial and fungal infection, and depletion of essential

growth-promoting components such as L-glutamine or growth factors. The cells will be discarded if they are found to be contaminated.

Mycoplasma infections are generally difficult to detect because these intracellular pathogens cannot be observed by standard light microscopy. Mycoplasma contamination was checked using a fluorescence microscopy-based commercial detection kit following the manufacturer's instruction (MycoFluoro™ Mycoplasma Detection Kit, Molecular Probe, USA). Figure 5.11(a) shows the cells seeded on a microscopy slide and the sample was tested to be negative where only the nuclei was stained and appeared bright blue in Figure 5.11(b).

(a) Cells seeded on a microscopy slide. (b) Stained nuclei appeared bright blue.

Figure 5.11: Mycoplasma testing of culture.

A positive culture will show particulate or filamentous fluorescence around the cell nuclei. A heavy infection of mycoplasma will stain in the inter-cellular spaces (see Figure 5.12).

(a) Cells seeded on a microscopy slide (b) Inter-cellular spaces were stained.

Figure 5.12: Positive sample of mycoplasma testing.

5.5.3 Fabrication of control collagen scaffolds

Freeze-dried collagen scaffolds of similar dimensions (15mm diameter; 4mm thickness) were fabricated to serve as the control specimens. Briefly, dissolved collagen (Sigma) at a concentration of 1%w/v was cast into a 15mm diameter dish. The scaffolds were then frozen at -20°C for 8 hours and dried using a freeze dryer (Christ, alpha 1-2) with the ice condenser temperature at -55°C .

5.5.4 Seeding of SaOS-2 cells on scaffolds

The scaffolds were sterilized with filtered 70% ethanol at room temperature for 3 hours. The ethanol was removed by immersing in sterilized phosphate-buffered saline (PBS) on an orbital shaker (100rpm) for 8 hours. The scaffolds were then soaked in the culture medium for at least 8 hours. A total of 1.6×10^6 cells/ml were seeded onto each scaffold.

5.5.5 Static culture

Cells were seeded on the scaffold and allowed to attach for 2 hours in a 24 wells plate. 1ml of medium was added into each well after 2 hours. The scaffolds were maintained in the incubator at 37°C and 5% CO_2 -humified atmosphere for a total culture period of 7 days. The medium was changed every 3 days. Samples were taken out after 1, 3 and 7 days of cultivation for proliferation, morphological and histological analysis.

5.5.6 Direct perfusion culture

The static culturing technique has been the *de facto* method in many cell culture experiments. However, in this research a dynamic culturing strategy was developed to maximize the potential of the collagen scaffolds. Detail of the design and development of the bioreactor is presented in Chapter 6.

5.5.7 Cell growth proliferation assay

Proliferation of cells in the scaffolds was tested using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) cell growth proliferation assay after 1, 3 and 7 days of cultivation. The MTT Cell Proliferation Assay (Sigma, USA) is a colorimetric assay system, which measures the reduction of a tetrazolium component

into an insoluble formazan product by the mitochondria of viable cells. The insoluble dark blue formazan salt can therefore give a qualitative assessment of the location of cells within the scaffold. The formazan salt is soluble in detergent reagent to yield a colour solution. The intensity of colour produced is directly proportional to the number of viable cells; hence, quantitative result of cell proliferation can be obtained.

After incubation of the cells with the MTT reagent (5mg/ml) for 4 hours, a detergent solution was added to lyse the cells and to solubilize the colored crystals. The samples were read using a spectrophotometer (Benchmark Plus, Bio-Rad, USA) at a wavelength of 570 nm. The spectrophotometer measures quantitatively what fraction of light passes through the colour solution. Results were expressed as the MTT absorbance unit vs days in cultivation. The result can be converted to the number of cells according to a cell standard calibration curve (see Figure 5.13).

Figure 5.13: Cell standard calibration curve.

The cells were released from flask by scraping and resuspended at 1×10^6 cells/ml. Serial dilutions of cells in culture medium were prepared from 1×10^6 to 1×10^3 cells/ml. 100 μ l of the dilutions were filled into wells of a microtiter plate in triplicate. Three control wells of medium alone were included to provide the blanks for absorbance readings. The cells were incubated for at least 8 hours to recover from handling.

25 μ l of MTT reagent was added to each well, including controls. The plate was returned to cell culture incubator for 4 hours. When the purple precipitate became clearly visible under the microscope, 100 μ l of Detergent Reagent was added to all the wells, including controls. The plate was left with cover in the dark for overnight at room temperature.

The absorbance was measured in each well, including the blanks, at 570 nm in a spectrophotometer. The average values were determined from triplicate readings and the average value for the blank was subtracted. The graph of absorbance against number of cells/ml was plotted.

5.5.8 Scanning electron microscopy analysis

The morphologies of seeded and unseeded scaffolds were investigated by scanning electron microscopy. Samples were fixed overnight with 2.5% glutaraldehyde in PBS at 4°C. Following fixation, the samples were then dehydrated with graded ethanol (from 70% to 100%) and critical point dried (CPD030, Bal-Tec, Germany). The samples were then sputter-coated with gold–palladium (JFC-1600, JEOL, Japan) and images were collected using SEM (JSM-5600LV, JEOL, Japan).

5.5.9 Histological analysis

For histology studies, samples were processed using a tissue processor (Leica TP1020, Leica Microsystems, Germany) and embedded in paraffin according to the manufacturer's instructions. 7 μ m sections were cut with a Microtom (Leica RM2125RT, Leica Microsystems, Germany) and fixed onto silane-coated glass slides (Sigma, USA) with water. Haematoxylin and eosin staining was performed according to the steps shown in Table 5.2. Hematoxylin is a dark purplish dye that stains the

chromatin (nuclear material) within the nucleus, leaving behind a deep purplish-blue colour. Eosin is an orangish-pink dye that stains the cytoplasmic material including connective tissue and collagen.

Steps	Action	Time
1-3	Xylene (Sigma), 3 changes	5 minutes each
4-5	100% ethanol, 2 changes	5 minutes each
6	95% ethanol	5 minutes
7	70% ethanol	5 minutes
8	Tap water	Rinse
9	Gill's 2 hematoxylin solution (Sigma)	12 minutes
10	Tap water	Rinse
11	1% Acid alcohol (99ml of 70% alcohol mixed with 1ml of concentrated hydrochloric acid)	Rinse
12	Tap water	Rinse
13	Scott solution (1L of deionized water containing 2g potassium bicarbonate and 20g magnesium sulphate)	5 minutes
14	Tap water	Rinse
15	Eosin Y solution, alcoholic (Sigma)	3 dips
16-17	Tap water, 2 changes	Rinse
18	70% ethanol	30 seconds
19	95% ethanol	30 secomds
20-21	100% ethanol, 2 changes	1 minutes each
22-24	Xylene , 3 changes	1, 3 and 5 minutes each

Table 5.2: Staining protocol.

5.5.10 Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was performed using the two-population Student's *t*-test. The significant level was set at *P*<0.05.

5.6 Summary

In this chapter, the indirect approach which uses a sacrificial mould to transfer the morphology of the mould to the collagen, has been described. Two different drying methods namely, critical point drying and freeze-drying were investigated and the scaffold morphology obtained was compared.

The accuracy of the mould was investigated, as it would directly affect the morphology of the resultant scaffold. Characterization analyses on collagen included thermal analysis and Fourier Transform Infrared (FTIR). The internal morphology of the scaffolds was studied using the SEM. The porosity of the collagen scaffolds was measured using two different techniques, namely the liquid displacement technique and the gas displacement technique.

Osteoblast-like SaOS-2 cells were used to evaluate the in vitro performance of the collagen scaffolds. Two different culturing techniques namely the static and the direct perfusion culture were described. The performance of the scaffolds was indicated by MTT-based cell proliferation assay, SEM analysis and histological analysis for the evaluation of cell proliferation and distribution in scaffolds.

For the dynamic culturing strategy, the design and development of the direct perfusion bioreactor will be described in Chapter 6.

CHAPTER 6

BIOREACTOR DESIGN

6.1 Direct Perfusion Bioreactor

As stated in section 2.5, several bioreactors have been developed for use in tissue engineering. There have been many efforts in developing bioreactor customized to different types of scaffolds for *in vitro* application. The more common ones are the spinner-flask, rotating vessel, direct perfusion chambers and bioreactors that apply controlled mechanical forces on the tissue constructs [12].

However, there are few reports on the application of direct perfusion bioreactor for compliant scaffold such as collagen scaffolds used in this research. The low mechanical strength of the scaffold poses a challenge on the design of the bioreactor. Furthermore, direct perfusion bioreactor that is commercially available in the market is limited and relatively costly. It is the objective of this research to design and develop a customized bioreactor for the collagen scaffolds. Bioreactors that utilize direct perfusion chambers have a few advantages over the others.

Direct perfusion bioreactors have the ability to enhance diffusion and apply mechanical forces on the construct at the same time. Media can be perfused directly through the interconnected pores of the scaffolds. This would allow nutrients and oxygen to penetrate through the porous scaffolds and bring about superior cell growth throughout the scaffolds. Mechanical stimulation in this case can be provided in the form of shear stress, by varying the flow rates at which the medium is perfused.

Another benefit of this system is that the continuous influx of fresh medium removes any detached dead cells, eliminating any undesirable cell apoptosis signals. The proposed bioreactor would be used for the culture of collagen scaffolds with internal channels.

6.1.1 Specification and requirement

There are a few requirements that the proposed bioreactor design needs to take into consideration:

(1) As the bioreactor is intended to culture tissue constructs using collagen scaffold in the shape of a disc, the chamber designed would have to be circular in shape in order to hold the scaffolds firmly.

(2) The bioreactor has to be put into an incubator during cell culture, as it will provide the necessary environment (eg. The right amount of carbon dioxide and oxygen, appropriate temperature, and a clean environment). Thus the bioreactor's size has to be small enough to fit into the incubator. A typical incubator would be 640mm wide, 690mm deep and 1900mm tall. The overall dimensions of the bioreactor would have to be much smaller to fit into the shelves of the incubator.

(3) As it is with all equipment used in tissue engineering, the bioreactor has to be made of non-toxic and autoclavable materials. Thus polymeric materials would be chosen as the primary material to fabricate the bioreactor.

(4) Considering the flow direction of the media, it is best that a top-down flow design be adopted in order to minimize the presence of bubbles at the surface of the scaffold. The flow through the chamber must be designed in such a way that the medium will flow through the scaffolds instead of bypassing around them to ensure the idealized shear stress generated at every plane.

(5) In order to be efficient in providing the effects of direct perfusion appropriately, the flow circuit system must be able to deliver a steady and consistent flow of medium to the constructs cultivated. This can be achieved with a peristaltic pump and an efficient flow circuit design.

(6) The bioreactor should be reasonably easy to be operated and provide room for alterations or additions to its functionality.

6.2 Design and Development

A preliminary design was proposed considering the specifications and constraints discussed in Section 6.1.

6.2.1 Culture chamber design

A schematic of the design of the culture chamber is shown in Figure 6.1.

Figure 6.1: Cross-sectional view of assembled chamber.

The culture chamber consisted of a cylindrical cap and a base. The scaffold retainer and the filter mesh held the collagen scaffold in place in the chamber. The retainer can be machined easily into different diameter for scaffold of different size.

The flow of the medium would be in a top-down fashion (in the direction of the blue arrows as shown). Gaskets were placed at the top and bottom of the retainer to prevent fluid leakage.

The chambers were assembled into place with the top and base plates. This was done by tightening the stainless steel bolts and nuts, holding the plates together.

6.2.2 Modular components development

Each culture chamber is made up of six individual components as shown in Figure 6.2. The components are easy to fabricate and assemble. The inlet and the outlet of the chamber were machined to be fitted with standard Luer fittings.

Figure 6.2: Modular components of culture chamber.

The detail on the design and material selection for each component is presented in the following paragraphs.

(a) Culture chambers

The chambers of the bioreactor must be made of materials that are chemically inert, light weight, possess good tensile strength, easily formed into shapes and relatively inexpensive. As such, a polymeric material, Polypropylene (PP) was selected. The same material was selected for the filter mesh. The design of the base and the cap is shown in Figure 6.3 and Figure 6.4 respectively.

Figure 6.3: Base of the chamber.

Figure 6.4: Cap of the chamber.

(b) Gaskets

Gasket should be sufficiently ductile to be pressed tightly against the base chamber (see Figure 6.5). Silicone that is chemically inert and autoclavable was used for this purpose.

Figure 6.5: Gasket.

(c) Scaffold retainer

The scaffold retainer should be autoclavable, chemically inert, easily machined into desired dimension and low in cost. Acrylic was selected based on these criteria.

(d) Filter mesh

The filter mesh acted as a base to support the compliant collagen scaffold to prevent the collapse of scaffold under the fluid pressure build up in the culture chamber. The PP filter mesh selected is autoclavable and can be cut easily into required dimension. A mesh with pore size of $500\mu\text{m}$ was selected for ease of fluid flow.

(e) Top and base plates

Stainless steel was selected for the top and base plates based on the high mechanical strength. The top and base plates were completely excluded from the culture medium flow path, eliminating any possible contamination from the metal ion. The designs for the plates are shown in Figure 6.6 and Figure 6.7.

(a)

(b)

Figure 6.6: (a) Dimensions and (b) 3-D view of the base plate.

(a)

(b)

Figure 6.7: (a) Dimensions and (b) 3-D view of the top plate.

6.2.3 Flow circuit design

The flow circuit assembly can be separated into six individual flow paths as shown in the schematic in Figure 6.8. Each individual flow path can be operated on its own, hence enhancing the ease of testing the chambers one at a time. The medium was drawn from the reservoir using a peristaltic pump. It was pumped through the tubings, into the chambers, through the scaffolds and then returned to the reservoir. As such, the culture medium can be continuously recirculated through a closed loop. The medium reservoir used consisted of an autoclavable polycarbonate square bottle and a leak proof PP closure fitted with 3 silicone ports. Two ports were used as feeding and receiving ports while a filter was attached to the remaining port to ensure sufficient air exchange in the medium. Luer connectors were used in the flow circuit that would reduce the complexity of the set up.

Figure 6.8: Schematic Diagram of Flow Circuit Assembly.

6.2.4 Continuous flow control

A peristaltic pump (ISMATEC®, Ecoline VC-360) was selected to deliver the flow through the bioreactor. The pump delivered fluid using a rotor with a number of rollers attached to the external circumference of it. A flexible tube was fitted inside a circular pump casing. As the rotor turned, the part of tube under compression closed thus moving the fluid through the tube. The flow was always contained inside the sterile tube, hence the risk of contamination from the pump was eliminated.

The peristaltic pump delivered a fixed volume of fluid through the entire flow circuit. The speed setting on the pump was controlled by a 2-digit potentiometer that ranges from 01 to 99. Experiments were conducted to determine the flow rate delivered via the selected tube at the setting of 01 to 15. The pump setting in the higher range of 15-90 was not investigated as the resultant fluid shear stress might be too high and can be detrimental to the scaffold and the cells. At each setting, the fluid delivered in 1 minute was collected using a measuring cylinder. The step was repeated 6 times and the readings were averaged. The data collected is shown in Table 6.1.

Pump setting	Flow rate (ml/min)
01	2.87
02	5.83
03	10.50
04	13.58
05	15.00
06	18.00
07	22.50
08	25.50
09	32.25
10	37.17
11	39.83
12	44.42
13	49.08
14	51.25
15	52.33

Table 6.1: Flow rate of different pump speed setting.

The graph shows the relationship of the pump setting to the flow rate delivered via the selected Pharmed[®] Ismaprene tube of inner diameter 3.2 mm (see Figure 6.9). It was selected because it is autoclavable, non-toxic and non-hemolytic.

Figure 6.9: Flow rate relative to the pump setting.

6.3 Assembled Bioreactor

The assembly of the fabricated chambers and plates corresponded to the proposed design, as it had a tight-fit, flow-through configuration, and was ready for the application of direct perfusion flow (see Figure 6.10).

Figure 6.10: Assembled bioreactor.

The flow circuit assembly designed was capable of delivering a steady, consistent, accurate and repeatable flow. This was done with the use of a peristaltic pump, appropriate fluid circuit connectors and tubings.

6.4 Summary

There have been many efforts in developing bioreactor customized to different types of scaffolds for *in vitro* application. However, there are few reports on the application of direct perfusion bioreactor for compliant scaffold materials such as collagen used in this research. The low mechanical strength of the scaffold posed a challenge to the design of the bioreactor. To overcome this challenge, the culture chamber was designed carefully with scaffold retainer ring and filter mesh to hold the scaffold in place for successful perfusion of medium through the circuit.

The bioreactor worked as a re-circulating loop, which overcame diffusion limitations of nutrients and oxygen and hence enhanced the overall mass transfer through the scaffolds. At the same time, it was capable of providing mechanical stimuli in the form of pressure and shear stress. These in turn could be modulated by the flow rates of culture medium.

CHAPTER 7

RESULT AND DISCUSSION

In this chapter, the results of the various experiments are presented. Collagen scaffold without internal channels were fabricated using conventional freeze-drying method and evaluated to serve as a benchmarking to the RP collagen scaffold with internal channels. The internal pore architecture achieved was analyzed and is discussed in Section 7.1.

The resolution of the printed part would directly affect the resolution of the final scaffold since the morphology of the scaffold will take on the geometry of the mould. Therefore, the resolution of the machine was investigated to evaluate the performance of this process in Section 7.2. Two different drying methods namely, critical point drying and freeze-drying were investigated and the scaffold morphology obtained is reported in Section 7.3. The criteria of the comparative study include dimensional shrinkage, degree of open porosity and patency of interconnected 3-D channels as designed. The physical and chemistry properties of the RP collagen scaffold were studied and are discussed in Section 7.4.

The performance of the cell-seeded collagen scaffolds was evaluated under two different culturing techniques, namely the static culture and the direct perfusion culture. Details are presented in Section 7.5 and 7.6.

7.1 Evaluation of Conventional Freeze-dried Collagen Scaffold

Scanning Electron Microscopy (SEM) observations revealed that highly porous foam-like interconnected networks were formed in the fabricated collagen scaffold after conventional freeze-drying process (see Figure 7.1).

Figure 7.1: Internal pores of conventional freeze-dried collagen scaffold.

Differences in pore size and surface morphology can be explained on the basis of ice crystal growth. Ice crystal growth and hence pore size is a function of the temperature gradient generated by freezing rate. Fast freezing at low temperature induces cracking, formation of uniform small channels, and the production of a fibrous structure. Slow freezing at higher temperatures results in nonuniform and large pores. The possible cases of interconnectivity inside the foam are illustrated in Figure 7.2. The continuity of the interconnection is not guaranteed and might be blocked or isolated due to collapsed pore and certain spot with lower heat transfer rate.

Figure 7.2: Interconnection of pores.

A problem encountered in the application of the freeze-drying method to prepare the scaffolds was the occurrence of surface skin (see Figure 7.3). Prior to freeze-drying, the collagen scaffold was put in a freezer to induce the formation of ice crystal in the solution. The free surface of the solution lost heat to the surrounding directly and hence solidified more rapidly than the bulk material in the solution. As a result, smaller ice crystals were formed at the surface layer.

During the freeze-drying stage, the ice crystal sublime to vapour phase when the molecules have enough energy to break free. According to Shuttleworth [200], the total free energy per mole of small crystals is greater than that of large crystals due to the larger surface to volume ratio. This translates into a greater distortion during sublimation and the surface layer of the collagen scaffold is not rigid enough to resist the interfacial tension. Thus, the porous structure collapses and dense skin layers occur in the fabricated scaffolds.

Figure 7.3: Skin layer of conventional freeze-dried scaffold.

This layer of surface skin is an undesirable feature induced by the freeze-drying fabrication method. As shown in the plan view of the skin layer (see Figure 7.4), it covers up the labyrinth of the interconnected pores underneath it, rendering the scaffold impermeable to fluid at the surface level and hence exerting a negative impact to the diffusion efficiency through the scaffold.

Figure 7.4: Plan view of the skin layer.

The non-homogeneous morphology can be explained by the unequal cooling rate that exists in the solution during the freezing phase. During the freezing process, ice crystals nucleate and grow. The portion of solution in contact with the container interface experiences higher rate of heat transfer as compared to the bulk material, hence, resulting in the smallest ice crystal growth. The cooling rate decreases as the distance from the interface increases, causing a gradient of freezing rate throughout the entire cross section of the sample (see Figure 7.5). The sample solidifies under time and space dependent freezing conditions leading to an inhomogeneous pore structure.

Figure 7.5: Heat transfer during freezing.

Manipulating the pore morphologies requires a comprehensive understanding of the ice crystal growth kinetics in the solution. A range of parameters such as the polymer concentration, type of solvent and the cooling rate, can influence the freezing dynamics. Researches have shown that different pore morphology can be achieved by controlling the freezing regime during fabrication or by changing the freezing rate. However, the optimization process of these interrelated parameters to achieve acceptable levels of control is time consuming and expensive. The indirect RP technique investigated in this project would enable direct control on the pore morphology of the scaffold.

7.2 Characterization of RP Fabricated Mould

7.2.1 Resolution of printed mould

Figure 7.6 shows a printed mould after the removal of support material (see Figure 7.6). The overall dimensions were $11 \times 11 \times 3 \text{ mm}^3$. The branching internal channels were designed to have the width of $600\mu\text{m}$, $400\mu\text{m}$ and $200\mu\text{m}$ respectively as shown in Figure 7.7.

Figure 7.6: Photograph of printed mould.

Figure 7.7: Internal branching channels.

Qualitative examinations showed that at larger width, the printed lines had parallel sides and the corners of the edges are sharp (see Figure 7.8). This showed that the printheads were aligned correctly and the volume of the droplets deposited was consistent.

Figure 7.8: Qualitative view of machine accuracy.

However, observations on smaller features of the printed part showed that the corners were rounded and the edges had uneven sides (see Figure 7.9). This could be due to insufficient accuracy of the support structure deposited since the channels were at elevated level and must be supported by the support material.

Figure 7.9: Accuracy of small feature.

Quantitative investigation on part accuracy was performed using 3 printed samples. The measurements were taken at suitable interval. 12 readings were taken for each channel size (see Figure 7.10).

Figure 7.10: Measurements taken on channels width of (a) 600 μm (b) 200 μm

The results are presented in Figure 7.11. From Figure 7.11 it is clear that the performance of the machine improved as the feature size increased. The discrepancy generated was rather consistent as shown by the consistent distance between the two curves in Figure 7.6.

Figure 7.11: Comparison of designed and experimental wall thickness.

The capability of the machine can be influenced by a number of factors including the calibration of the printheads, control of precise volume deposition and alignment of the printhead. The resolution can be enhanced by calibrating the machine using the specific calibration plot.

Similarly, quantitative investigation was also performed on the accuracy of layer thickness (see Figure 7.12). The machine was able produce very high layer thickness accuracy due to the layer-by-layer milling operation that eliminates any accumulative error. The actual layer thickness was measured as 51.8 μ m compared to the specified value of 50.8 μ m. The error recorded was 1.96%.

Figure 7.12: Measurements taken on layer thickness.

7.2.2 Accuracy of internal channels mould

Figure 7.13 shows a printed mould after the removal of support material. Its overall dimensions were 15mm diameter and 4mm thickness. The branching internal channels were designed to have the width of 500µm.

Figure 7.13: Different views of the CAD design and the RP printed mould.

The 3D inkjet printer was able to produce intricate structure with fine features. The mould was fabricated at a specified layer thickness of 0.0508mm. The diameter of each channel in CAD design was 500µm. Measurements from the SEM images showed that the diameter of the printed channels was $573\pm23\mu\text{m}$, which is 14% bigger than the designed value.

7.2.3 Surface finish of mould

The mould shown in Figure 7.14 exhibited characteristic burr formations on the surface as a result of the droplet-based manufacturing process. The droplets spread and flatten into a half spherical cap upon impact to the substrate. Hence, the edge defined by the material had a convex and rounded profile. This was predicted and explained in chapter 3.

Figure 7.14: Surface finish of mould.

The convex profile increased the surface area of the scaffold. The increment of surface is believed to be useful for cell attachment. Furthermore, in the case of a scaffold carrying bioactive element such as HA, the additional exposed surface could help the dissolution rate of such bioactive element, enhancing the bioactivity of the scaffold [104].

7.3 Comparison of Drying Techniques

7.3.1 Dimensional shrinkage of scaffolds

The dimensional shrinkages of the scaffolds were plotted and are shown in Figure 7.15.

Figure 7.15: Dimensional shrinkage of scaffolds. (* indicates significantly higher)

The dimensional shrinkage induced by CPD was significantly higher than that of the FD process ($p < 0.05$). The diameter of the CPD scaffolds shrank significantly higher at $(28 \pm 6)\%$ as compared to FD scaffolds that experienced shrinkage of $(16 \pm 3)\%$ ($p = 0.0005$). The thickness of the CPD scaffolds was reduced significantly with a shrinkage of $(46 \pm 4)\%$. In comparison, the amount of thickness shrinkage induced by the FD process was much smaller ($p < 0.0001$). The thickness of the FD scaffolds was found to have shrunk by $(15 \pm 5)\%$. This result obtained corresponded to a recent reported research which showed that a scaffold of 1.0%w/v collagen dispersion shrank more than 70% in volume percentage after CPD [71]. Hence, the FD process is more favourable since the amount of shrinkage induced was approximately half of the CPD process. Furthermore, the shrinkage induced by the FD process showed a more favourable homogeneous profile as compared to the nonlinear shrinkage of CPD scaffold.

7.3.2 Effect of critical-point drying

In this study, it would have been a convenient choice to use CPD as the drying method since one of the principal steps in the process requires the use of ethanol to remove the channel mould. However, the SEM analysis showed that the CPD process was unable to produce a foam-like porous structure with high porosity. There was no visible open porosity as the pores were surrounded by thick fibril wall (Figure 7.16). The mean diameter of the irregular pores was measured as $53\pm 23\mu\text{m}$. The thickness of the wall could be as thick as $40\mu\text{m}$ (Figure 7.17). The permeability of the scaffold would be affected significantly by the loss of interconnected porosity and the increased thickness of pore wall.

Figure 7.16: Critical-point dried scaffolds.

Figure 7.17: Thick fibril wall of pores.

7.3.3 Effect of freeze-drying

Material to be freeze-dried must first be adequately frozen. Freezing the dissolution resulted in the production of ice crystals that grew and forced the collagen into the interstitial spaces, thus aggregating the collagen. The kinetics of the freezing mechanism was governed by heat transfer in the dispersion. The freezing point of the solvent, ethanol was very low at -114°C [201]. Therefore it was necessary to replace the ethanol with distilled water prior to freezing the construct again at -20°C .

The freeze-drying process was able to reproduce the design configuration of interconnected 3-D internal channels as shown in Figure 7.18. SEM analysis showed that the volume around the hollow channels was a porous foam structure that will increase the surface area for cell attachments (see Figure 7.18).

Figure 7.18: Through channel with pores around.

7.3.4 Indirect fabrication technique of collagen scaffold

The experimental results showed that freeze-drying was found to be more suitable as it induced less shrinkage and reproduced the design morphology accurately. Similar results have been reported in other researches [202, 203]. Dagalakakis *et al* reported that collagen-glycosaminoglycan (GAG) membranes, initially swollen in distilled water or saline, shrank linearly to no less than 94% of original dimension after FD but was reduced to 75% after CPD. They concluded that CPD resulted in significantly more shrinkage and a lower mean pore size than either one of the two freeze drying

procedures used [202]. In another study by Trieu *et al*, which researched on an alternative method in preparing PVA hydrogel, the CPD technique also showed a collapsed fibrillar structure with much less porosity [203].

Previous report has shown that scaffolds with internal channels were produced using CPD [71]. However, the configuration of the reported channels was different from this study. The mould design used in this study involved a configuration of 3D channels that extended to different planes of height. CPD used in the previous study was not sufficient to reproduce the channels morphology in this report, as the non-linear shrinkage induced by the process would produce channels of different sizes at different locations if not blocked completely.

The process developed in this research was able to reproduce the design configuration of interconnected 3-D internal channels (see Figure 7.19).

Figure 7.19: Collagen scaffold with internal channels.

7.4 Characterization of RP Collagen Scaffold

7.4.1 Overall internal morphology of RP collagen scaffold

Microscopy analysis shown in Figure 7.20 revealed that the volume around the branching hollow channels was a porous foam structure that will increase the surface area for cell attachments. The arrows in Figure 7.20 indicate the location of the internal channels.

Figure 7.20: Micrograph of collagen scaffold with internal channels.

The thickness of the scaffold produced was slightly larger than the 4mm specified in the CAD design. The distance between the horizontal channels was measured as 4.3mm and 4.2mm as compared to the designed value of 4mm (see Figure 7.21).

Figure 7.21: Distance between horizontal channels.

7.4.2 Microscopy analysis of RP collagen scaffold

The morphology of the RP scaffold was further analyzed with SEM. It was observed that the internal channels were surrounded by a porous foam structure, a resultant from the freeze drying process (see Figure 7.22 and Figure 7.23).

Figure 7.22: Internal channel through the RP scaffold surrounded by porous volume.

Figure 7.23: Internal channel through the RP scaffold surrounded by porous volume.

The channel's diameter obtained was measured as $437\pm100\mu\text{m}$. The final channel's size was slightly smaller than the intended design of $500\mu\text{m}$ diameter. A closer examination revealed that the channels were defined by a layer of skin, which was the resultant product of the freeze-drying process. In this application, these skin layers

have helped to sustain the morphology as well as the patency of the 3-D channels in the scaffolds (see Figure 7.24). The wall layer was approximately 30-40 μ m thick.

Figure 7.24: Wall layer of internal channel.

The mean diameter of the pores surrounding the through channels was $180\pm63\mu$ m (see Figure 7.25). The data are presented in Appendix C.

Figure 7.25: Internal pores of collagen scaffolds.

Pore size has been observed to influence adhesion, growth and differentiation of a wide variety of cell types. Researches have shown that a cell does exhibit selectivity on pore size according to specific cell types [65, 70]. Lu *et al* suggested that the critical pore size is said to be greater than 150 μ m to favour new bone formation [62].

Another group reported that the optimal pore size for regeneration of bone was 100-600 μm [68]. Therefore, the collagen scaffolds fabricated in this research are suitable for the regeneration of bone.

The current scaffold design provides the opportunity to manipulate the morphology of the scaffold at three different scales namely (1) the macroscopic scale, defined by the outer shape of the scaffold; (2) the intermediate scale, which are the interconnected channels that play important roles in mass and flow transport; (3) the microscopic scale, defined by the pores from sublimation of ice crystal (see Figure 7.26). The design of the scaffold at each scale can be modified in order to create the environment for the desired cell growth behaviour.

Figure 7.26: Morphology of the scaffold at different scales.

7.4.3 Porosity

The porosity of the scaffolds was measured using two techniques to investigate the porosity of the scaffolds.

Firstly, the liquid displacement method measures the open porosity of the scaffolds [192, 193]. This technique has been accepted as the established method for measuring porosity of compliant scaffolds that are unable to withstand the high pressure applied in mercury intrusion porosimeters that range from 0.5 to 60,000 psi [193]. This method is relatively simple and easy to compute. However, it is manual-based and the accuracy can be compromised due to the absorption of liquid by the scaffold as well as the small volume of the scaffold.

On the other hand, the gas pycnometer works by measuring the amount of gas displaced by the solid phase of the sample and hence, the solid volume of the scaffold is obtained. This method requires minimum manual work and the scaffold can be measured in the dry state. Consequently the data obtained is considered more accurate. The results obtained from both techniques are presented in Figure 7.27.

Figure 7.27: Comparison of techniques in porosity measurement.

The porosity measured using the liquid displacement technique was 0.5 for both groups of the scaffolds. The porosity of the control collagen scaffolds (0.71 ± 0.02) is higher than the RP scaffolds (0.64 ± 0.03) according to the gas displacement method ($p < 0.05$). This could be due to the extra processing steps required when transferring the mould channels morphology into the collagen scaffolds. Some of the pores might have collapsed during the ethanol removal step.

The lower value of total porosity in the RP fabricated scaffolds did not significantly affect the *in vitro* performance of the scaffold. This was because there are more vital factors such as the interconnectivity of the pores and the size of the interconnection as shown by Bignon *et al* [59]. Recent work had reported that narrow pore interconnection inhibited bone tissue differentiation in the pores [204]. The interconnectivity of the pores and the size of the interconnection were well controlled in the RP fabricated scaffolds.

7.4.4 Fourier transform infrared spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) is an analytical technique used to identify organic materials. This technique measures the absorption of various infrared light wavelengths by the material of interest. These infrared absorption bands identify specific molecular components and structures.

The FTIR spectra of collagen in Figure 7.28 show the confirmation of the presence of the protein backbone. A characteristic peak at $3380\text{--}3400\text{cm}^{-1}$ was observed. This band corresponded to the amine --NH stretching vibration. Distinct peaks were observed around $1500\text{--}1600\text{cm}^{-1}$, which was the amide I and amide II band. The amide I band peaks occurred due to predominant carbonyl C=O stretching [205], while the amide II band occurred due to both amines --NH and C-N stretching [205]. Amide III band was seen at around 1200cm^{-1} .

Figure 7.28: FTIR analysis of collagen scaffold.

The amide I band has been reported to be associated to the triple helical structure of collagen [206]. The preservation of triple helical structure indicated reduced immunogenicity of the collagen scaffold [207].

The original functional features of the collagen were shown to remain intact. Hence, it was concluded that the processing steps did not denature the material or impair the biocompatibility of the collagen.

7.4.5 Differential scanning calorimetry analysis

Differential scanning calorimetry (DSC) measures the temperatures and heat flow associated with transitions in materials as a function of time and temperature. The temperature of the sample crucible was measured with respect to the reference crucible, and as a result, any exothermic or endothermic reactions were detected, even if there is no weight loss or weight gain.

Upon heating, a large exothermic peak was found at approximately 90°C. The peak reached a maximum at 94.37°C. This is the maximum rate of decomposition under the given condition. From the slope of the peak, it can be shown that the melting temperature of collagen was 47°C. The area under the curve shows the heat capacity of the material and was measured to be 360J/g by using a feature in the software (see Figure 7.29).

Figure 7.29: DSC analysis of collagen scaffolds.

7.5 Static Culture of the Collagen Scaffolds

7.5.1 Proliferation of cells

Proliferation of cells in scaffolds was tested using MTT-based cell growth proliferation assay after 1, 3 and 7 days of cultivation. The results presented in Figure 7.30 show significantly higher cell proliferation in RP scaffold with internal channels on day 1 and day 3, and slower cell proliferation on day 7.

	Control (A ₅₇₀)	RP (A ₅₇₀)
Day 1	0.53±0.015	0.71±0.19
Day 3	0.58±0.028	1.19±0.15
Day 7	2.03±0.22	2.13±0.16

Figure 7.30: Proliferation of cells on collagen scaffold under static culture.

The number of attached cells on RP fabricated scaffold was found to be significantly higher on Day 1 ($p<0.05$). The number of cells on control and RP scaffolds was 4.74×10^4 cells/ml and 6.73×10^4 cells/ml respectively. It could be due to the fact that the delivery of cell-containing medium into the scaffolds was aided by the presence of internal channels.

SEM analysis on the cross section of the scaffold showed presences of cells in the interconnected channels as indicated by the arrows in the enlarged view shown in Figure 7.31.

Figure 7.31: Cells attached in channels.

The number of cells in RP scaffold has almost doubled after 3 days of cultivation. This proliferation rate is comparable to the rate achieved during 2-D flask cells

cultivation. The number of cells in RP scaffolds was found to be significantly higher than the control scaffold ($p < 0.0001$). There were 5.27×10^4 cells/ml on control scaffold, while the number of cells on RP scaffolds was one magnitude higher 1.20×10^5 cells/ml.

A delayed cell growth pattern was observed in the control collagen scaffolds. By day 7, the results showed that the difference narrowed in the cell number between the two groups of scaffolds due to the rapid proliferation of SaOS-2 cells in the control collagen scaffolds. The high proliferation rate of cells in control scaffolds was possible due to the low cell density in the control scaffold by day 3. The number of cells recorded on control scaffold was 2.11×10^5 cells/ml.

For RP scaffolds, the cell proliferation rate slowed down after 7 days in the culture. A continuous model was used to calculate the theoretical number of cell in scaffold. The classical exponential growth law was applied by assuming that the cell growth rate is proportional to the number of cells in scaffold [208].

$$\frac{dN_{cell}}{dt} = kN_{cell} \quad (\text{Equation 8.1})$$

Where N is the number of cell and k is the proportional constant.

The number of cells can be solved as follows:

$$N_{cell}(t) = N_0 e^{kt} \quad (\text{Equation 8.2})$$

Where proportional constant, $k = 0.231 \text{ day}^{-1}$ by assuming a doubling time of 3 days and initial cell number, $N_0 = 6.73 \times 10^4$ cells/ml as recorded.

The number of cells after 7 days in cultivation was recorded as 2.22×10^5 cells/ml which was 34.5% lower than the calculated value of 3.39×10^5 cells/ml. This could be explained by the nutrient diffusion limitation elicited by the high cell density observed at the surface of the scaffold. A dense layer of cells was formed on the scaffold surface after 3 days of cultivation as shown in Figure 7.32.

Figure 7.32: Dense layer of cells attached on the surface of scaffold.

Further proliferation of cells would require the cells to migrate into the scaffold in 3 dimensions. However the culture environment at the surface of scaffold was more favourable compared to the interior, as there was limited medium flux through the scaffold in unstirred medium. The dense layer of cells on the scaffold surface further depleted the local concentrations of nutrient and oxygen in the interior of scaffold. As a result, the continuous proliferation of cell in the RP scaffolds was inhibited. Similar phenomenon had been reported in other research where cells were unable to penetrate into the scaffold under static culture [24]. Cells were found mostly attached to the outer surface of the scaffold.

The performance of the RP scaffold could be enhanced when cultured under appropriate conditions to induce and support the migration of cells into the scaffold. A dynamic culture technique that perfuses the culture medium through the scaffold was investigated for this purpose. Results will be discussed in Section 7.6.

7.5.2 Distribution of cells

The penetration depth of cells into the scaffolds after 7 days in static culture was observed and measured from scanning electron micrographs. The micrographs showed that a dense layer of cell attached at the exterior of the scaffold. The channels incorporated though the scaffold served as flow conduits that delivered cells and fresh medium fluid into the central region of the scaffold.

As a result, accelerated cell growth pattern was observed and the cells were able to attach and proliferate even at the inner region of the RP scaffold (see Figures 7.33). Arrows in the figure indicate the locations of cells that have penetrated into the scaffold.

Figure 7.33: Cross-section of cell-seeded RP scaffolds.

In the control scaffolds, it was observed that most of the cells attached at the outermost layer of the scaffold, forming a dense layer as shown in Figure 7.34.

Figure 7.34: Dense layer of cells at exterior of control scaffold.

Observation on the cross-sectional micrographs of the control scaffold revealed that, compared to the RP scaffolds, fewer cells were found in the interior area (see Figure 7.35).

Figure 7.35: Cross-section of cell-seeded control scaffolds.

The maximum depth below the surface with cells was measured as $15.28 \pm 1.81 \mu\text{m}$ for the control collagen scaffold. The result obtained was significantly higher ($p < 0.001$) in the RP fabricated scaffold with internal channels where cells were found up to a depth of $47.7 \pm 3.22 \mu\text{m}$. This represented a more than three fold increase in the depth that cells have penetrated into the scaffold. The results are presented in Figure 7.36.

Figure 7.36: Maximum penetration depth of cells in scaffold under static culture.

The results shows that scaffolds with high porosity alone did not guarantee cells penetration depth, as the proliferation of cell into the scaffold would depend on the ease of medium diffusion into the scaffold. In addition, diffusive limitations may also inhibit the efflux of metabolic wastes produced in the scaffold interior. The incorporated channels have been proven to enhance the distribution of cells through the scaffold.

7.5.3 Morphology of cells

The appearance of cells varied with different topography of the scaffold surface. Reported studies had demonstrated that SaOS-2 cells displayed different morphology on different surface topography. Furthermore, the study also reported that apart from the surface characteristics such as micro and macro pore size, the presence of an interconnecting system also had a significant impact on the target cells [198]. It was observed that the cells attached on a flat surface displayed a wide spreading morphology, which is the characteristic of an osteoblastic cell (see Figure 7.37)

Figure 7.37: Cells attached on RP scaffolds.

On an uneven surface, the cells had long extension and conformed to the uneven surface topology of the scaffolds (see Figure 7.38). Lamellipodia extensions at the periphery of the cell are clearly shown in Figure 7.39. These extended cell structure served as 'legs' for better adhesion on scaffold and played an important role for subsequent motility during the migration stage.

Figure 7.38: Cells conformed to uneven surface.

Figure 7.39: Lamellipodia extensions at the periphery of the cell.

7.5.4 Histological analysis

Cells were found to have attached on the surface of the control collagen scaffold as shown in Figure 7.40(a) and (b). Collagen structures appeared in pink while cell bodies and nuclei were stained in dark purple. A thin layer of cells nucleus was observed.

(a)

(b)

Figure 7.40(a) and (b): Histological sections of control scaffolds.

For RP scaffolds (see Figure 7.41(a) and (b)), cells were observed at the interior of the scaffold. It can be shown that there are several layers of cells on the surface of the scaffold.

(a)

(b)

Figure 7.41(a) and (b): Histological sections of RP scaffolds.

7.6 Direct Perfusion Culture of RP Collagen Scaffolds

7.6.1 *In vitro* application of bioreactor

The bioreactor chamber set up and the culture medium reservoir were placed in an incubator (see Figure 7.42 and Figure 7.43). Non-permeable PVC tubing with Luer ends connected the culture chamber to the peristaltic pump. The PVC tube can be slotted through the soft seal of the door of the incubator without collapsing.

Figure 7.42: Bioreactor set-up.

Figure 7.43: Bioreactor in the incubator.

Cells were seeded on the scaffold and allowed to attach for 2 hours. Medium with 1ml was added into each well after 2 hours. The cell-seeded scaffolds were transferred to the bioreactor after 4 hours of static incubation. The bioreactor was maintained in a

5% CO₂-humified atmosphere incubator at 37 °C during operation. 250ml of medium was circulated through the circuit. Half the volume of the reservoir was changed every 2 days. Samples were taken out after 1, 3 and 7 days of cultivation for proliferation and morphological analysis.

7.6.2 Effect of pore structure on resistance to flow

Direct perfusion culture experiments were conducted on conventional freeze-dried collagen scaffolds and RP fabricated collagen scaffolds. Experimental results showed that the resistance to flow was higher for the conventional freeze-dried scaffolds. The scaffolds deformed and collapsed during flow perfusion experiments.

The internal pore structure of the conventional freeze-dried scaffold is shown in Figure 7.44. The pores were found to have formed in a layered fashion in the transverse direction, leaving membrane-like walls between the layers of pores with small interconnection between the pores. The flow path for the medium through the scaffold was tortuous and hence, fluid pressure was built up in the scaffold as well as in the culture chamber with the continuous inflow of fluid. The scaffold was compressed in the culture chamber, blocking the exit and the medium was not flowing through the chamber as it should be.

Figure 7.44: Layered pores wall blocked the flow path.

Flow through experiments were successfully conducted using RP fabricated collagen scaffolds. The data collected is shown in Table 7.1. This indicated that the permeability of the RP scaffold was higher than the control scaffold. The flow path throughout the scaffold was sufficient in channelling the influx and efflux of the fluid circulated in the bioreactor circuit.

Pump setting	Flow rate (ml/min)
01	2.90
02	5.63
03	10.47
04	13.67

Table 7.1: Bioreactor flow through experimental data set.

7.6.3 Analytical modelling of fluid shear stress

The hydrodynamics in the direct perfusion bioreactor can be analyzed using a simplified model for the flow through the scaffold (see Figure 7.45).

Figure 7.45: Modelling flow through scaffold in culture chamber.

The scaffold cultured was cylindrical in shape with a diameter of d_{sc} . Assuming flow was distributed uniformly across the scaffold surface, the mean velocity of the internal fluid flow through the pores, V_m , can be calculated as follows [20]:

$$V_m = \frac{Q}{A\phi} \text{ (m/s)}$$

The fluid shear stress on the wall of the channels, τ , is equal to the wall shear stress experienced by the attached cells, given as follows [20]:

$$\tau = \frac{8\mu V_m}{d_{ch}} \text{ (Pa)}$$

The residence time represents the average length of time the culture medium remained in the scaffold. The residence time of medium through the scaffold is given by

$$T = \frac{Vol}{Q} \text{ (min)}$$

Where Vol is the volume of the scaffold.

Reynolds number of the flow, $Re = \frac{\rho V_m L_{sc}}{\mu}$

Where L is the characteristic length of scaffold in the direction of flow, hence, $L_{sc} = 4\text{mm}$.

Operating conditions of the bioreactor is summarized in Table 7.2:

Parameters	Symbol	Value
<u>Physical properties of scaffold</u>		
Diameter	d_{sc}	15 mm
Length	L_{sc}	4 mm
Diameter of pores	d_{ch}	500 μm
Porosity	ϕ	0.5
<u>Working fluid properties</u>		
Dynamic viscosity	μ	0.001 kg/ms
Density	ρ	1000 kg/m ³
<u>Operating parameters</u>		
Flow rate per scaffold	Q	1.90 ml/min
Mean flow velocity	V_m	0.04 cm/s
Shear stress	τ	0.15 dyn/cm ²
Residence time	T	0.40 min
Reynolds number	Re	0.20 (Laminar flow)

Table 7.2: Operating conditions of bioreactor.

7.6.4 Effect of direct perfusion on scaffolds

It was observed that the collagen scaffold, which is compliant and low in mechanical strength, appeared slightly compressed when retrieved from the bioreactor. Digital micrographs were taken to compare the scaffolds after 3 days of cultivation under direct perfusion culture and static culture (see Figure 7.46).

Figure 7.46: Effect of direct perfusion on RP collagen scaffold.

The height of the scaffold cultured in the bioreactor was reduced to approximately 1.2mm after 3 days of direct perfusion (see Figure 7.46(a)). The hydrostatic pressure in the culture chamber had compressed the collagen scaffold. The thickness of the collagen scaffold cultured statically in 24 wells plate was measured to be 2.27mm (see Figure 7.46(b)).

The scaffolds shown in Figure 8.46 were incubated with MTT for visualization of the distribution of cells. Areas populated by cells were stained dark purple due to the formation of the formazan crystals. The distributions of cells appeared more homogenous and populated a bigger area in the scaffold cultivated in the direct perfusion bioreactor (see Figure 7.46(a)).

Figure 7.46(b) shows uneven distribution of cells in scaffold. Most of the stained areas were concentrated at the periphery of the scaffold.

7.6.5 Proliferation of cells

Proliferation of cells in RP scaffolds was tested using MTT-based cell growth proliferation assay after 1 and 3 days of cultivation.

The results presented in Figure 7.47 show significantly higher cells proliferation in RP scaffolds cultured in the direct perfusion bioreactor as compared to static culture.

	Static Culture (A ₅₇₀)	Direct Perfusion Culture (A ₅₇₀)
Day 1	0.71±0.19	1.38±0.09
Day 3	1.19±0.15	2.72±0.83

Figure 7.47: Proliferation of cells in RP scaffolds under direct perfusion culture.

Preliminary results showed that the use of bioreactor had enhanced the proliferation of cells on the scaffolds. Significantly higher cell density was recorded in direct perfusion cultured scaffolds compared to the static cultured ones. The difference was almost two folds for both time points.

The scaffold cultured in the bioreactor was able to sustain higher cell density due to the continuous re-circulation of the medium flow. The diffusion barrier through a 3-D construct was overcome in this culturing technique. Medium containing growth-limiting factor such as nutrient and oxygen was delivered directly through every

connected pores and channels to the cells' surfaces for effective exchange and waste removal. The shear stress exerted on the cells was estimated as 0.15 dyn/cm^2 at a pre-determined medium flow rate of 1.90 ml/min . The shear stress did not damage the cells attached as this magnitude of shear stress was more than one magnitude lower than the reported value of $8\text{--}30 \text{ dyn/cm}^2$ for bone tissue under interstitial flow [209].

However, the collagen scaffolds were found to have deformed and disintegrated after 7 days of direct perfusion. This showed that the applied flow rate of 1.90 ml/min was unfavourable to the compliant collagen scaffolds for extended period. Further experiments were necessary to determine the optimum working flow rate for long-term cultivation of the RP scaffolds.

The overall results showed that the potential of the RP scaffold with internal channels could be maximized through the coupling with a direct perfusion bioreactor.

7.7 Summary

This study has successfully displayed an active control of the internal morphology of the collagen scaffold. Channels of less than 0.5 mm diameter were incorporated throughout the scaffold successfully at predetermined locations and configuration. These channels had shown to be supported by a layer of 'skin', which was the resultant product of the freeze-drying process. In this application, these skin layers had helped to sustain the morphology as well as the patency of the 3-D channels in the scaffolds.

In vitro cell culture experiments were performed on the control and RP collagen scaffolds. These cell-seeded constructs were cultured under static condition. Accelerated cell growth pattern was observed in the RP collagen scaffolds. It is believed that the channels incorporated through the scaffold served as flow conduits that delivered cells and fresh medium fluid into the central region of the scaffold. As a result, accelerated cell growth pattern was observed and the cells were able to attach and proliferate even at the inner region of the scaffold. This result demonstrated that the fully interconnected channels have helped to enhance the mass transfer efficiency

through the scaffold. Enhanced mass transfer is vital for sufficient nutrient delivery and waste exchange in order to sustain the viability of high cell density in the scaffolds. Furthermore, the distribution of cells was found to be more homogenous throughout the thickness of the RP scaffold.

A customized direct perfusion bioreactor was developed to further demonstrate the potential of the RP collagen scaffold with internal channels. The bioreactor allowed the direct flowing of medium through the scaffold, thus overcoming the diffusion limit occurred in static culturing technique while applying fluid shear stress on the cells. Preliminary results showed that the use of bioreactor had enhanced the proliferation of cells on scaffolds. Significantly higher cell density was recorded in direct perfusion cultured scaffolds compared to the static cultured controls.

CHAPTER 8

CONCLUSIONS AND FUTURE WORKS

8.1 Conclusions

This research explored the potential of using RP technology to fabricate TE scaffold via an indirect method, which uses a sacrificial mould to transfer the morphology of the mould to the biomaterial. A 3-D inkjet printer, T612TM, was selected to fabricate the sacrificial mould.

There are several key contributions from this research:

Firstly, the RP process using the 3-D inkjet printer was studied thoroughly to appreciate the capability of this process in the fabrication of tissue engineering scaffold. A comprehensive mathematical model has been established to relate the operating parameters of the RP process to the part dimensions.

Secondly, a novel fabrication process was developed successfully to transfer the RP part architecture to shape the internal morphology of collagen scaffold. Collagen scaffold with branching interconnected internal channels was fabricated.

Thirdly, a customized direct perfusion bioreactor for the cultivation of compliant scaffold was developed successfully. The proliferation of cells on RP collagen scaffolds cultivated in the direct perfusion bioreactor was evaluated.

The contributions are further elaborated in the following sections.

8.1.1 Mathematical model of 3-D inkjet printing

A mathematical model was developed for the 3-D inkjet printing process so as to provide physical insight to the process. The model was useful in the prediction of printing performance and investigation of the effect of specific variables. The

machine's performance is important in order to achieve the predetermined morphology and resolution of the mould utilized in this indirect fabrication technique of collagen scaffold.

Theoretical development was presented to illustrate the phenomena of liquid droplet formation, ejection, and impact on the substrate. According to the model presented, the layer thickness was affected by the ratio of frequency over printhead velocity. The thickness increased as printing frequency increased, or when the printhead moved at a slower speed. Surface roughness of the part produced was found to be dependent on the layer thickness as well.

8.1.2 Development of RP collagen scaffold

Conventional fabrication methods offer limited control over the pore architecture of a collagen scaffold. Furthermore, the continuity of the interconnection is not guaranteed and might be blocked or isolated due to the collapsed pores at certain spot with lower heat transfer rate. In this research, a method was developed successfully in fabricating collagen scaffold with predefined 3-dimensional networks of internal channels through the use of an indirect rapid prototyping technique. This novel fabrication process was able to replicate a configuration of 3-D channels that extended to different planes of height in the scaffold and the patency of these channels was assured. The resulting scaffold contained networks of internal hollow channels with porous foam-like structure occupying the surrounding volume.

The current scaffold design provides the opportunity to manipulate the morphology of the scaffold at three different scales namely (1) the macroscopic scale, defined by the outer shape of the scaffold; (2) the intermediate scale, which are the interconnected channels that play important roles in mass and flow transport; and (3) the cellular scale, defined by the pores from sublimation of ice crystal.

The design of the scaffold at each scale can be modified in order to achieve the desired cell growth behaviour. Parameters that can be modified include the overall dimension, shape, dimension, distribution, configuration as well as the orientation of the channels. With the advancement of computer-aided design, graded internal

channel mould design can be generated and scaffolds with functionally graded internal morphology can be fabricated with this method.

8.1.3 Development of direct perfusion bioreactor

A customized direct perfusion bioreactor for the cultivation of compliant scaffold was developed successfully to demonstrate the potential of the RP collagen scaffold with internal channels. The bioreactor is able to overcome the nutrient diffusion limit commonly observed in static culture. Mechanical stimulation on the cells was provided in the form of shear stress, by varying the flow rates at which the media is perfused.

The bioreactor was made of non-toxic and autoclavable materials. The flow circuit system designed was able to deliver a steady and consistent flow of medium to the scaffolds cultivated. Preliminary experiments had shown the successful application of the bioreactor *in vitro*.

8.1.4 *In vitro* performance of RP scaffold and bioreactor

The networks of internal channels had shown to enhance the proliferation of cells and the cellularity of the scaffold. *In vitro* static experiment evaluations showed significantly more cells attached to scaffolds with internal channels compared to control freeze-dried collagen scaffolds. Micrograph and histological analysis revealed that these cells were distributed more homogenously throughout the RP scaffolds.

The proliferation of cells on RP scaffolds was further enhanced by coupling it with the direct perfusion bioreactor. Significantly higher cell density was recorded in direct perfusion cultured scaffolds compared to the static cultured ones. The combination of a RP fabricated collagen scaffold and the direct perfusion bioreactor had shown to be able to maximize the proliferation of cells in scaffold and enhance homogeneous distribution of cells.

8.2 Future Works

8.2.1 Further development of RP collagen scaffold

The novel method developed in this research could be adapted to accommodate other polymeric biomaterials such as chitosan, PCL, PLLA and etc. The reported process had excluded high temperature, therefore, the bioactivity of the scaffold can be further enhanced by addition of bioactive element such as HA or biomolecules.

The collagen scaffolds fabricated possess low mechanical strength. The mechanical strength of the scaffolds can be improved by incorporating load-bearing elements such as ceramic or polymeric component to expand the functional application of the scaffold.

For further investigation on the design of collagen scaffold, channels of different configuration can be generated and scaffolds with different internal morphology can be fabricated with the improved method developed. This would enable future investigation into the growth behaviour of cell in response to different channels morphology.

The surface roughness of the RP-fabricated scaffolds should be investigated in detail. There is a need to understand how significant the effects of surface finish resulting from the RP process on enhancing the bioactivity of the scaffold. This can lead to a better design of the scaffold and the control of the RP process to maximize the performance of the collagen scaffold.

8.2.2 Development of bioreactor

The direct perfusion bioreactor had shown very promising result in the cultivation of collagen scaffolds. However, the design of the bioreactor can be further enhanced to incorporate a pressure valve so as to prevent the compression of scaffold in cultivation. The fluid circuit can be modified to accommodate sampling ports. Sample of culture medium can be collected through these ports for routine checking on the physiology condition of the cells seeded. Examples of analysis are pH level, glucose level, oxygen tension level and etc. Further cell experiments should be conducted to investigate the long-term application of the bioreactor.

8.2.3 Further characterization of cell-seeded scaffold

This thesis had emphasized on the characterization of cell proliferation and distribution of cells in scaffolds. Further characterizations analysis such as alkaline phosphatase (ALP) activity can be performed in order to assess the functionality of the cells in scaffolds. ALP activity is a marker of early osteoblastic differentiation. ALP can be measured colorimetrically and is based on the conversion of *p*-nitrophenyl phosphate into *p*-nitrophenol in the presence of alkaline phosphatase.

Histological staining other than Hematoxylin and Eosin for cell visualization can be explored too. Other staining analysis includes *von kossa* for visualization of mineralized bone.

8.3 Publications

This work has generated a number of journal and conference publications, listed as follows:

8.3.1 International refereed journal papers

1. **Rapid prototyping in tissue engineering: challenges and potential**, (2004) Yeong WY, Chua CK, Leong KF, Chandrasekaran M., Trends in Biotechnology, 22, 643-652.
Impact factor = 7.955; citations = 27.
2. **Indirect fabrication of collagen scaffold based on inkjet printing technique** (2006) Yeong WY, Chua CK, Leong KF, Chandrasekaran M., Lee MW, Rapid Prototyping Journal, 12, 229–237.
Impact factor = 0.814; citations = 1.
3. **Comparison of drying methods in the fabrication of collagen scaffold via indirect rapid prototyping** (2006) Yeong WY, Chua CK, Leong KF, Chandrasekaran M., Lee MW, Journal of Biomedical Materials Research: Part B - Applied Biomaterials, *in press*.
Impact factor = 1.621; citations = N.A.

8.3.2 Conference papers

1. **Development of scaffolds for tissue engineering using a 3D inkjet model maker** Yeong WY, Chua CK, Leong KF, Chandrasekaran M, Lee MW, International Conference for Virtual and Rapid Prototyping, Leiria, Portugal, Oct 2005. Awarded Best Paper at the conference.
2. **Rapid prototyping in tissue engineering: A state-of-the-art report** Chua CK, Yeong WY, Leong KF, International Conference for Virtual and Rapid Prototyping, Leiria, Portugal, Oct 2005.
3. **3D inkjet printing for rapid prototyping: Development of a physical model** Yeong WY, Chua CK, Leong KF, Chandrasekaran M, Proceedings of the 3rd International Conference on Materials Processing for Properties and Performance, Singapore, Nov 2004.
4. **Collagen scaffold with internal channels cultured in a direct perfusion bioreactor**, Yeong WY, Chua CK, Leong KF & Chandrasekaran M, 3rd International Workshop on Biomodelling and Bioprinting, Singapore, May 2006. Awarded Consolation Prize for student poster.

8.3.3 Journal papers in preparation

1. **Proliferation of SaOS-2 cells in collagen scaffold with internal channels fabricated by indirect rapid prototyping technique**, to be submitted to Tissue Engineering.
2. **Collagen scaffold with internal channels cultured in a direct perfusion bioreactor**, to be submitted to Biotechnology and Bioengineering.

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APPENDIX A

Technical Specifications of T612

Physical			
Build area	X: 30cm	Y: 15cm	Z: 15cm
Unit dimensions	W: 71cm	D: 50cm	H: 50cm
Unit weight	44kg		
Room temperature	16°C to 27°C		
Humidity	40% to 60%		
Current at voltage	10A at 230V		
Model Characteristics			
X, Y, Z accuracy	±0.001 inch per inch		
Z-axis build layer	0.013mm to 0.076mm		
Surface finish	32 to 63 micro-inches (RMS)		
Minimum feature size	0.254mm		
Droplet size	0.076mm		
ModelWorks Requirements			
Configuration	Microsoft Windows ME/NT/2000 or XP		
Processor	400MHz Pentium		
RAM	64Mb		
Hard disk space	500Mb available		
CAD file input	*.STL and *.SLC		

APPENDIX B

Information of SaOS-2 Cell Line

Supplier	ECACC
Catalogue no.	89050205
Cell line name	Saos-2
Keywords	Human primary osteogenic sarcoma
Description	<ul style="list-style-type: none">• Reported to have been derived from an 11-year-old female Caucasian.• Treatment RTG, methotrexate, adraimycin, vincristine, cytoan, aramycin-C.• HLA cell line phenotype: A2, 3;Bw16, w47.
Species	Human
Tissue	Bone
Morphology	Epithelial-like

APPENDIX C

Pore Size Measurement

The mean pore size of the scaffolds was determined by analyzing of the SEM images. Twelve apparent pores were measured per image. The pores were measured in a perpendicular direction through the long axis and the minor axis. The average value was then calculated and taken as the mean pore size.

Pores	Long axis (μm)	Minor axis (μm)
1	203	131
2	301	159
3	155	150
4	352	178
5	267	174
6	203	179
7	250	74
8	208	15
9	188	65
10	235	91
11	103	146
12	245	141
13	167	154
14	257	249
15	155	89
16	213	258
17	247	169
18	257	105
19	118	125
20	223	113
21	127	130
22	143	264
23	170	141
24	203	230
Mean	208 ± 60	153 ± 55
Overall Mean	180 ± 63 μm	