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**NANYANG
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**DEVELOPMENT OF FUNCTIONALLY GRADED
BIOMATERIAL SCAFFOLDS
USING SELECTIVE LASER SINTERING**

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SCHOOL OF MECHANICAL AND AEROSPACE ENGINEERING

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Abstract

Tissue Engineering (TE) aims to create biological substitutes to repair or replace failing organs or tissues due to trauma or ageing. One promising approach in TE is to grow cells on biodegradable scaffolds, which act as temporary supports for the cells to attach, proliferate and differentiate; after which the scaffold will degrade, leaving behind a healthy regenerated tissue. Tissues in nature, including human tissues, exhibit gradients across a spatial volume, in which each identifiable layer has specific functions to perform so that the whole tissue/organ can behave normally. Such a gradient is termed a functional gradient. A good TE scaffold should mimic such a gradient, which fulfils the biological, mechanical and anatomical requirements of the target tissue.

Thus, the design and fabrication process of such scaffolds become more complex and the introduction of computer aided tools combined with rapid prototyping (RP) techniques will lend themselves well to tackle these issues. The challenge in fabricating functionally graded scaffolds (FGS) using RP techniques lies in the development of suitable computer aided systems to facilitate the FGS design. What have been missing are the appropriate models that relate the scaffold physical properties, e.g. pore size and porosity, to the biological and mechanical requirements for the regeneration of the target tissue. The establishment of these relationships will provide the foundation to develop better computer aided systems to help design a suitable customized FGS.

Computer Aided System for Tissue Scaffolds (CASTS) is a system consisting of a parametric library of polyhedral unit cells that can be assembled into a tissue scaffold. The existing CASTS system only allows the design of scaffolds with uniform porosity. Thus, this research focuses on the development of automated system based on CASTS for designing customized FGS intended for bone applications. The first step to the establishment of such system was the derivation of relationships between scaffold structure, porosity and compressive stiffness for polyhedral CASTS scaffolds fabricated using SLS. Based on the modified rule of mixtures, the compressive stiffness of the CASTS scaffolds could be predicted when the scaffold porosity is known.

The next step was the integration of the porosity-stiffness relationships into the automated system algorithms. Such algorithms for designing FGS, both radial gradients for long bone applications and linear gradients for short or irregular bone regeneration have been achieved. A new cellular unit for constructing radial FGS, which was the unit cylinder, was introduced. A complete model relating the FGS parameters to the unit cell dimensions and assembly has also been established. The feasibility of the automated FGS system was verified using case studies of femur and mandible FGS. Selective Laser Sintering (SLS) was used to fabricate the scaffolds, and polycaprolactone (PCL) and polycaprolactone/hydroxyapatite (PCL/HA) were used as the scaffold materials. This automated FGS system is beneficial in aiding tissue scaffold designers in creating scaffolds with gradients mimicking the target organ. With the inputs of the scaffold stiffness variations and dimensions, the system alleviates the tedious drawing routines and the dependency on the user's computer skill.

Finally, *in vitro* studies investigating the effects of varying scaffold porosity on cell proliferation and matrix mineralization were carried out. It was found that varying scaffold porosity varied scaffold surface area, which in turn affected the cell proliferation and function. The cell number on the scaffolds corresponded proportionally with the surface area of the scaffold, as well as the calcium content of the extra cellular matrix (ECM). Three-dimensional scaffold structure was proven to be a necessity for cell function, such as ECM deposition and mineralization, reflected by the significantly higher calcium deposit-to-cell number ratio of all scaffolds compared to those of two-dimensional polystyrene well. As time progressed, tetrazolium dye MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) results and scanning electron microscopy (SEM) observation showed that the cells were able to attach and proliferate, forming multiple cell layers and infiltrating the scaffold's micro pores. This implied that SLS-sintered scaffolds could provide a favorable environment for the cells to attach, proliferate and deposit mineralized matrix for a prolonged period.

Chapter 1 – INTRODUCTION

1.1. Background

The loss or failure of organs or tissues due to trauma or ageing is one major concern in human health care as it is a costly and devastating problem [1-2]. This problem has led to the development of tissue engineering (TE), which aims to create biological substitute to repair or replace the failing organ and tissues [2-3].

One of the approaches in TE is to seed appropriate cells on degradable biomaterial scaffolds to culture them to form new tissues that can replace the damaged ones [1, 4-5]. Biodegradable scaffolds are highly engineered structures that act as a temporary support for cells to facilitate the regeneration of the target tissue. For example: TE scaffolds for bone regeneration are made of polycaprolactone (PCL) or polylactic acid (PLA) and are seeded with osteoblasts, which secrete and mineralize bone extra cellular matrix (ECM) [6]. Likewise, in cartilage regeneration, biomaterials such as collagen or PLA are formed into scaffolds to be seeded with chondrocytes, which secrete the ECM constituting the articular cartilage [7]. The challenge in such an approach is to design and fabricate customizable biodegradable scaffolds with desirable properties that can promote cell adhesion, support cell growth, proliferation and differentiation, and eventually the formation of the extra cellular matrix (ECM) for the tissues [4, 8].

The desirable properties or requirements of TE scaffolds can be categorized into three perspectives, namely anatomical, biological, and mechanical requirements [9-10]. Anatomically the overall/external shape of the scaffold should fit the defect site so as to anchor the scaffold firmly to the target organ [11]. Besides the external architecture, different cell types prefer different scaffold internal architectures, namely pore size, porosity, and also surface properties of the scaffold [12]. These constitute the biological requirements. Further critical requirements are the scaffold mechanical properties, such as stiffness and strength, which should match those of the target organ. Mechanical properties are very important, particularly in the regeneration of load-bearing organ, i.e.

bone, to avoid stress-shielding and to bear physiological load [13]. Often, these requirements result in conflicting demands, for example: the high porosity that is favored for cells proliferation and nutrient transport is detrimental to the required scaffold stiffness. Thus, balance and trade-offs are required in designing suitable scaffold internal architecture to satisfy these requirements in order to better accommodate the growth of the cell. These suggest that the control over scaffold design parameters such as pore size and porosity is critical.

Besides the design of internal architecture, scaffold properties are highly influenced by the processing technique and the biomaterial that is used to make the scaffold. There are a variety of processing methods to fabricate TE scaffolds [8]. Unfortunately, most of these processes have limitations in controlling the pore size, porosity, pore interconnectivity, and in turn mechanical properties, which are the critical aspects of a scaffold [6, 14]. Recently, rapid prototyping (RP) techniques have been found to be promising in TE application because of their flexibility and outstanding manufacturing capabilities. These distinct advantages include its ability to fabricate TE scaffolds with controllable micro- and macro-architecture [14-16].

The control over the scaffold microstructures is important because human organs or tissues are observed to be functionally graded, i.e. they possess some kind of gradient adapted to their function and different layers have their own specific functions and are important for the tissue or organ to function properly. They may consist of more than one cell type or phenotype and different types of cell have shown to prefer specific scaffold environment for the cells to grow, proliferate and function well [9-10]. Take the example of skin, which consists of two layers (epidermis and dermis). The epidermis is composed primarily of keratinocytes, which is tough and impermeable to toxic substances and harmful organisms and it also regulates the loss of water from the body. On the other hand the dermis is relatively acellular and mainly composed of collagen fibrils as ECM to provide strength, flexibility, and elasticity to the skin [17]. Another example is bone, which has structural and porosity/density gradient that causes the bone to possess

mechanical properties gradient, i.e. stiffness and strength. The stiffer part is for bearing the load and the less stiff and more porous part for absorbing strain energy [18].

In order to guide the formation of tissues with appropriate functional gradient, a successful scaffold is likely to have regions with different microstructures including different pore size and porosity [19]. There should also be an intermediate region between the two regions to allow for smooth transition to avoid scaffold delamination and also to better facilitate the stress transfer [20]. Such scaffold is called a functionally graded scaffold (FGS) since each region is uniquely related to the typical functions the cells in the specific region are performing and is meant to facilitate the cells in performing their functions [9].

Designing scaffold with functional gradient that fulfills anatomical, biological and mechanical requirements make the design stage of the scaffold complicated and tedious. To date, there are only a few Computer Aided Design (CAD) systems that are suitable for such design. One example of such system is the Computer Aided System for Tissue Scaffolds (CASTS) [11]. CASTS enables users to design customizable scaffold with ease. The essence of the system is a collection of cellular units that can be assembled together into three-dimensional scaffolds [21-22]. Users can acquire the defect image from a patient's body as an input to the system. After the cellular units assembly is ready, a Boolean operation is done between the defect image and the assembled cellular units. The result will be a scaffold consisting of stacked cellular units with the external shape of the patient's organ/tissue defect [11].

Nevertheless, the current CASTS system only allows the construction of scaffold with uniform pore size and has limitations to regenerate a heterogeneous tissue with functional gradient. Therefore, a system that allows designers to create FGS with ease is considered necessary. Such system will allow the design of FGS with CASTS system as the platform. The cellular unit can be selected to meet the variation of biological and mechanical requirements across the scaffold, while the scaffold customized external shape meets the anatomical requirement.

1.2. Motivation

From the native tissue point of view, there are three types of requirements, namely, biological, mechanical, and anatomical requirements. Biological requirements arise from the structures of the native tissues themselves, having different layers with unique functions of each layer for the tissue or organ to function normally. This can be found in both hard and soft tissues. Bone as a hard tissue possesses cortical and cancellous parts [23], while skin as a soft tissue consists of dermis and epidermis [17], and cartilage, also soft tissue, owns zonal gradients of ECM and chondrocytes phenotypes [24]. Thus, functionally graded scaffolds (FGS) in terms of pore size and porosity are needed to facilitate the cells to proliferate and differentiate in the appropriate layer to achieve healthy tissue regeneration.

From a mechanical standpoint, human tissues have different mechanical properties distributed spatially with respect to the anatomical site. Cortical and cancellous bones have different mechanical properties for smooth stress transfer functions, where the cortical bone acts as load bearer and cancellous bone as a load damper [18]. To temporarily bear the load and prevent stress shielding, the scaffold should be tailored to have corresponding mechanical properties, such as stiffness and strength, with the target bone, which can be achieved by varying the structure and porosity of the scaffold.

Last but not least, the external geometry and size of the scaffold should fit the tissue defect site in order for the scaffold to anchor onto the defect site properly [25]. This is the anatomical requirement.

Accounting for such complex requirements, FGS are needed to help regenerate defected tissues into healthy tissues. Some earlier works in the literature include FGS development for a single organ, such as bone or cartilage, and a combination of two organs, such as osteochondral regeneration [20, 24, 26]. The focus of bone FGS is the development of scaffolds with porosity and hence stiffness gradient mimicking the

bimodal structure of cortical and cancellous bone, where the more porous scaffold portion allows cells ingrowth, while the denser portion supports the physiological load [27-30]. On the other hand, in the case of cartilage regeneration, scaffolds with pore size gradient to a certain extent are able to control the ECM component gradient mimicking those of native cartilage in order to provide near frictionless articulating motion [31-32]. Another example is the osteochondral scaffold, which has pore size/porosity gradient fulfilling the requirements for both the bone portion and the cartilage portion [20, 33].

Currently, the challenges are the design and fabrication processes of such scaffolds. RP processes are preferred to conventional processes as the fabrication means due to the high degree of control and consistency over the scaffold macro and microarchitecture [14], including the fulfillment of the anatomical requirement. Such accuracy and consistency are due to the use of integrated CAD system with RP. However, fabricating FGS presents new challenges in automating the design process, as manual CAD designing of FGS will be a very complicated task.

In general, TE scaffolds are made up of small units of pores or fibers that are periodically repeated to form a 3D porous object [34]. Based on this concept, some CAD systems dedicated to aid/automate TE scaffold design have been developed. Some success evidences of such CAD systems can be found in the literature, for example, the works of Hollister *et al.* [35], Chua *et al.* [21-22], Cheah *et al.* [36], Wettergreen *et al.* [37-38] and Naing *et al.* [11]. Early efforts in the direction of FGS fabrication have also been made. One example is the scaffold library constructed by Wettergreen *et al.* [38], in which a common connector feature of a torus shape was added at every sides/facets of each cellular unit. This feature acted as a bridge to allow a smooth change between two different cellular units.

In this project, a system that can automate the FGS design process, both linear and radial gradient, is proposed. The summarized motivation of this project is shown in Figure 1.1.

Figure 1.1. Motivation of the research

1.3. Objectives

The objectives of this project are as follows:

1. To develop an automated system to design FGS based on CASTS for bone TE. The automated system includes both linear and radial gradients, where the linear FGS is meant for irregular or short bones regeneration, while the radial FGS is for long bone regeneration.
2. To study the effect of varying scaffold porosity on its compressive stiffness and strength and obtain the empirical relationships.
3. To study the effect of varying scaffold porosity on the osteoblast proliferation and mineral deposition.
4. To derive the relations between the parameters of FGS, integrating the porosity-mechanical properties relationships, and to develop the algorithm for designing linear and radial FGS.
5. To validate the feasibility of the integrated system using SLS, a powder based RP system, as a means of scaffold fabrication. Case studies are carried out by fabricating

bone scaffolds for bone parts such as jaw to represent the linear FGS and femur segment to represent the radial FGS.

1.4. Scope

This project includes:

1. Identifying and understanding the functional gradients across bone tissue, various scaffold requirements for bone tissue regeneration, as well as scaffold design and fabrication techniques.
2. Fabricating all configurations of the CASTS scaffolds using a suitable material using SLS. A biopolymer, polycaprolactone (PCL), and a biocomposite, PCL/hydroxyapatite (PCL/HA) have been chosen as the materials to fabricate the scaffolds.
3. Characterizing the mechanical properties, such as compressive stiffness and strength of the fabricated CASTS scaffolds and relating them to the scaffold porosity in order to implement the relationship into the FGS algorithm.
4. Studying the effect of varying scaffold porosity on the proliferation and mineralization of osteoblasts via *in vitro* experiments.
5. Investigating the structures of cellular units in CASTS in order to arrange the cellular units into scaffolds with porosity gradients. This includes developing algorithms and incorporating the algorithm into the CASTS system to automate the generation of FGS.
6. Designing and fabricating bone scaffolds for femur and mandible to validate the feasibility of the automated FGS system.

1.5. Thesis Organization

This report is organized as follows:

Chapter 1 gives an introduction to TE, including the requirements and desired properties of TE scaffolds, the need for functional gradients to mimic native organs, and also the

challenges in fulfilling the scaffold requirements. A solution to face the limitation is proposed.

Chapter 2 presents literature reviews of TE in general, biomaterials and fabrication processes of TE scaffolds. Functional gradients in natural tissues and the need to mimic such gradients are discussed, specifically in the case of bone TE. Following that, the desired TE scaffold characteristics are derived based on the functional gradients. Finally, CAD systems dedicated to aid the design of TE scaffolds are presented.

Chapter 3 describes the CASTS system, the CAD system dedicated for aiding scaffold design used in this project, the Selective Laser Sintering (SLS) system used to fabricate the scaffolds and the biomaterials used including polycaprolactone (PCL) and polycaprolactone/hydroxyapatite (PCL/HA). The rationale behind the usage of SLS as the fabrication means and PCL and PCL/HA as the biomaterials used are presented.

Chapter 4 discusses the development of the FGS automated system, including the various parameters of the FGS and the relationships among the mentioned parameters. The FGS automation algorithm, both linear FGS for short or irregularly shaped bones and radial FGS for long bone regeneration, are presented.

Chapter 5 details the experimental methods on scaffold fabrication and characterization, both in terms of physical characteristics and *in vitro* studies. The first section on scaffold fabrication explains the methods on finding the suitable SLS parameters to build TE scaffolds, both for PCL and PCL/HA. SLS samples are then characterized in terms of scaffold resolution and the surface roughness and the effect of varying scaffold porosity on its compressive stiffness and strength are investigated. The automated FGS system is validated by generating FGS samples based on human jaw and tibia and then fabricating the samples using the SLS. The *in vitro* study section involves cell culture protocols and cell seeding and observation methods, including the investigation of the effect of scaffold porosity on cell proliferation and mineralization.

Chapters 6 and 7 provide the results and discussions of the experiments detailed in Chapter 5. Chapter 6 presents the scaffold fabrication and characterization results, while Chapter 7 discusses the results obtained from the *in vitro* studies.

Finally, Chapter 8 concludes the report and suggests the future work for this project.

Chapter 2 – LITERATURE REVIEW

Tissue engineering (TE) is a multidisciplinary field, which involves life sciences (cellular and molecular biology) and principles of engineering (materials, chemical, and mechanical engineering) [1-4]. In order to achieve its goal, which is to create biological substitute for failing organs, one promising approach involves the use of a TE scaffold as a temporary support to initially assist the cell attachment, proliferation, and differentiation, after which the scaffold will degrade, leaving behind a healthy regenerated tissue [39-40]. Depending on the condition and the size of the defect tissue, one can use either the scaffold alone (acellular method) or in combination with cells and growth factor [41-43]. A schematic diagram of scaffold-guided TE is shown in Figure 2.1.

Figure 2.1. Schematic diagram of scaffold-guided tissue engineering (TE) [44]

In general, native tissues and organs are observed to be non-homogeneous and there exist natural functional gradients in their structures, as seen in the examples of bamboo, mollusk shells, tree trunks and human tissues such as skin, cornea, esophagus, bone, and

cartilage [17, 23, 32, 45-48]. Constituting the functional gradient, native tissue is typically composed of multiple differentiated layers, in which each layer has one or more specific functions to perform. Such tissue or organ is said to be functionally graded across the layers. Thus, a preferred TE scaffold should also be functionally graded mimicking the native tissue environment [49]. This is to facilitate the appropriately seeded cells to proliferate at the desired layer and to perform their functions in order to regenerate into healthy tissues. This can be observed and described from three perspectives: biological, mechanical and anatomical. Matching these perspectives will provide the favorable environment required for cell growth and proliferation for successful organ or tissue regeneration [25, 50]. Such scaffolds can be called functionally graded scaffolds or FGS in short [50].

As such, this chapter presents a review of various functionally graded structures evident in native tissues, as well as their implications on FGS design and development in terms of biological, mechanical and anatomical requirements (refer to Section 2.1). Existing works on computer-assisted design of FGS and the challenges identified in designing customized FGS are discussed in Section 2.2. These are followed by Section 2.3, which presents the various biomaterials for scaffold fabrication, and Section 2.4, which discusses the methods for fabricating the FGS, both conventional and rapid prototyping (RP) techniques. This chapter is then summarized in Section 2.5, reaffirming the identified research problem.

2.1. Functionally Graded Scaffolds (FGS)

The term “functionally graded” came from the field of material science to describe a material with varying structures or compositions distributed spatially over its volume, resulting in changes in the material properties [51]. As mentioned at the beginning of this chapter, the term FGS is used in TE field to call a scaffold with a structure/composition variation (e.g. pore size, porosity, or material variation) catering to the different functions each layer needs to perform [50]. In order to better understand the various aspects of FGS,

namely biological, mechanical and anatomical aspects, it is best to begin with an observation of the functional gradients existent in natural living tissues as follows:

2.1.1. Biological Requirements

The different layers of human tissue perform different roles in maintaining the organ functions. One layer may possess cell types or phenotypes that are different from other layers and has its own specific functions to enable the tissue or organ to function normally. For example, native articular cartilage has anisotropic cell distribution, from the articular surface to the part mating with bone. Articular cartilage can be classified into three zonal organizations according to the cell arrangement and the unique ECM production that is specific to their zonal organizations, namely, superficial (the one nearest to the articular surface), middle and lower (the one nearest to the bone) zones [52]. Chondrocytes, the cells that maintain and secrete the ECM of cartilage, appear more flattened in the superficial zone with collagen fibrils oriented parallel to the articular surface, while chondrocytes in the middle zone are more rounded with randomly oriented collagen fibrils. In the lower zone, chondrocytes have spherical shape with collagen fibrils oriented perpendicular to the articular surface (refer to Figure 2.2). These conditions contribute to the biomechanical functions of the articular cartilage [32]. The chondrocytes in the superficial surface are densely populated and secrete proteoglycan 4 (PRG4), molecules that may aid the surface lubrication. The density of chondrocytes decreases and the matrix secretion such as collagen II and aggrecan increases with increasing depth from articular surface. The increase in matrix components and the varying orientation of collagen fibers makes the compressive properties increase as it goes deeper from the surface, while the superficial zone, which is the softest, increases the contact area and distributes the load [53-54]. Woodfield *et al.* illustrated that a scaffold designed with pore size gradient could be used to affect the cell zonal arrangement and ECM distribution to a certain extent in constructing a tissue engineered cartilage [32].

Figure 2.2. Chondrocytes distribution and collagen fiber orientation in articular cartilage [32]

In terms of biological requirements, scaffold characteristics such as pore size and porosity play important roles in influencing cell growth, morphology, and phenotypic expression [55-56]. However, due to the differences in cell size and cell-matrix adhesion mechanism, different cells respond differently to the same scaffold microstructure and surface property [57-59].

While different cell types have different preference of scaffold pore sizes (summarized in Table 2.1), generally scaffolds with high porosity (around 90%) facilitate better cell growth, infiltration and ECM deposition [12]. Bose [60] and Bignon [61] have shown that the increase of porosity is accompanied by an increment in cell growth due to the effect of higher surface area per unit volume.

Table 2.1. The preferred scaffold pore sizes for different cell types

Cell type	Cell size (μm)	Preferred pore size (μm)
Hepatocytes	20–40 [62]	20 [8]
Fibroblast	20–50 [58]	90-360 [63]
Osteoblast	20–30 [64]	100-900 [8, 65]

For the regeneration of multiple tissues and tissue with functional gradient, scaffolds with pore size and porosity gradient will be critical [19]. For example, in bone-cartilage tissue

regeneration, more than one cell type is necessary. These different cell types have different environments *in vivo*, hence they need different scaffold pore sizes and porosities to better accommodate the proliferation and growth of the cells [20, 65-66]. In the work by Sherwood *et al.* [20], the porosity for the cartilage region was 90% with pore size of 106–150 μm , while the porosity for the bone region was 55% with pore size of 125–150 μm .

Another important biological requirement is the surface properties of the fabricated scaffold. It has been known that surface properties such as surface composition, surface roughness and hydrophilicity play an important role in regulating cell attachment, spreading and ECM deposition. It is also known that different cell types reacted differently to the same surface property [57]. In general, hydrophilic surfaces are preferred and coating a scaffold using suitable protein to improve its hydrophilicity contributed positively to cell attachment and proliferation [67-68]. Regarding scaffold surface roughness, it is believed that there exists a range of preferred surface roughness value for each cell type. Schneider *et al.* found that osteoblasts experienced increased mineralization when seeded on rough implant surfaces with microtopography relative to smooth tissue culture plastic [69]. However, the surface roughness value was not quantified in this work. Seeding human gingival fibroblasts on high purity aluminum oxide substrate with different surface roughness, Mustafa *et al.* found that the rougher the surface was, the more the cells attached to the substrate [70]. The range of surface roughness value experimented on by Mustafa *et al.* [70] was 2-90 nm.

2.1.2. Mechanical Requirement

Human body architectures have complex geometry with varying mechanical properties distributed spatially with respect to the anatomical site [23]. Using human bone as an example it can be observed that bone tissue is differentiated into two layers, namely cortical and cancellous/trabecular bone. The cortical part is the outer layer, which is rigid and dense, while cancellous bone (the inner layer) is a spongy honeycombed structure filled with blood vessels and bone marrow as shown in Figure 2.3. Bone is an optimized

structure in terms of strength and weight, i.e. bone's structure is porous and not all dense so that it is not too heavy for the vertebrates to carry and move about [23, 71].

Figure 2.3. Cortical and cancellous part of a bone [23]

As observed from the transverse section, human bone has a graded structure with varying porosity such that the strength and stiffness gradually become lower from the outer towards the inner section [23]. Particularly seen at the end of long bones, such as femur, there is a gradual porosity increase (from 3-16% in cortical bone [72-74] to 74-91% in cancellous bone [74-75]) in the cancellous-cortical transition [76]. Other type of bone, such as the cancellous bone at the mandible, can be classified into three different regions according to their compressive stiffness gradient, i.e. anterior (average stiffness of 67.48 MPa), middle (47.30 MPa), and distal regions (35.55 MPa) [77].

As such, a scaffold should have sufficient mechanical strength to keep its structural integrity until the new tissue regenerates, maintain the space for cell ingrowths and nutrient transport *in vitro* [16] and support physiological loadings *in vivo* [78]. The scaffold should match its mechanical properties to that of the native tissue, especially for load bearing tissue such as bone. Mismatch of mechanical properties between the scaffold and the bone causes stress shielding, which happens when the scaffold is stiffer than the target tissue and therefore it shields the bone from the load and causes bone resorption, or scaffold collapse/failure if the scaffold has lower strength or stiffness than the target bone [13].

Hence, to better suit its functions the scaffold needs to be modeled as a multilayer structure with varying mechanical properties across the scaffold. This can be done by varying the porosity across the scaffold, as porosity is a dominant factor in determining the scaffold mechanical properties, including strength and stiffness [61].

Besides the strength and stiffness of the scaffold, mechanical stimuli such as fluid shear stress or cyclic strain, which can be provided by a bioreactor, has been found to enhance protein expression and mineral deposition of bone cells (osteoblasts) [79-80]. After all, human bones adapt their structure and properties based on a variety of external loads that they carry, causing the collagen fibers in bone to be oriented according to the direction of the resulting stresses. This is supported by the evidence that such orientations are not found in fetuses [81]. The stiffness of ECM of the tissue affects its response to externally applied loads, which in turn affects the interstitial fluid flow, cell-cell distance and signaling and also ECM components gradients [82], both for hard tissue and soft tissue [83].

As a soft tissue, articular cartilage regeneration is known to be influenced by such mechanical cues. Generally, articular cartilage is loaded by compression loadings *in vivo*. Such mechanical loading affects changes at cellular level such as tissue deformation, fluid flow and nutrient and ion concentration gradients. Thus, compressive loadings promote chondrocytes viability, gene expression and the secretion of various ECM components such as collagen, proteoglycan and fibronectin in articular cartilage constructs [84]. It was also found that fluid flow in turn affected the mechanical properties of the chondrocytes cultured in scaffolds, where generally laminar flow improved the mechanical properties of the construct, in contrast to turbulent flow [84]. It appeared that the equilibrium modulus of the cartilage construct cultured in laminar flow was about four times the modulus of the constructs cultured in turbulent flow and static culture [84]. Besides, having fluid flow inside the scaffold would increase the effectiveness of nutrient and waste transport [85].

2.1.3. Anatomical Requirements

Anatomically, the external shape and size of the scaffold should be the same as those of the tissue defect so that the scaffold can fit and anchor well onto the defect site [25]. With appropriate external geometry and size of the scaffold, better fixation can be achieved, while facilitating better stress distribution at the interface between the surrounding tissue and the scaffold. Anatomical requirements can be fulfilled by utilizing dedicated CAD systems to aid the scaffold design process, for example: Computer Aided System for Tissue Scaffolds (CASTS) [11] and Computer-Aided Tissue Engineering (CATE) [86-87]. The general steps in obtaining the appropriate information for designing patient-specific scaffold are discussed in Section 2.2.

Summarizing the scaffold requirements from Sections 2.1.1-2.1.3, several important criteria for preferred tissue scaffolds [4, 15, 65] are listed as follows:

1. Having suitable physical characteristics and internal architecture such as pore size, porosity, pore interconnectivity, and also surface area to volume ratio. It has been shown that scaffold microstructure influences cell behaviors, such as cellular adhesion, proliferation, and migration [88-89]. There are optimal ranges of acceptable scaffold architecture suitable for cell growth, which is unique for different cell types [12, 90] (refer to Table 2.1).
2. Having sufficient mechanical properties that match the tissue at implantation site to support the newly formed tissue. By controlling the structure of the scaffold, the mechanical properties can in turn be controlled. It is important to match the mechanical properties, such as stiffness and strength, of the scaffold to those of the original tissue, especially for load bearing tissue such as bone in order to bear physiological load and prevent stress shielding [13, 78].
3. Having suitable surface properties, such as surface roughness, to facilitate the attachment of the cells, spreading, and also the function of the cells in secreting their ECM. It is stated in the literature that there exists an optimum range of surface roughness value and different cell types reacted differently to the same surface property [67-68, 70, 91-92].

4. Being made from materials that exhibit good biocompatibility with a predictable rate of degradation and produce non-toxic degradation products. The material used to create the scaffold should not raise significant adverse reaction (inflammation) or immune rejection from the host when implanted. The biocompatibility is affected by the processing techniques of the scaffold. When the fabrication technique makes use of emulsifiers, cross-linking agents, or organic solvents, these chemicals may not be completely removed from the scaffolds and could be toxic to the newly formed tissue as well as the surrounding host tissue, causing cell death or tissue necrosis [78].
5. Able to be shaped to fit into the defect site of the organ or tissue [11].
6. Able to be sterilized to prevent infections after implantation. The scaffold sterilization method should not significantly alter the chemical composition of the material from which the scaffold is made of, as it will affect its biocompatibility [78].

2.1.4. *Spatial Gradient in FGS*

For the convenience of this study, spatial gradients are categorized as either discrete or continuous gradients. Discrete gradient has multilayer structures with distinctive interfaces between the layers, while the continuous gradient has, as the name suggests, a continuous change in microstructure with spatial position (see Figure. 2.4) [51]. Concerns are raised over abrupt structural change in discrete gradients due to mismatches, incompatibility and stress concentration at the interfaces between the layers, which may cause delamination between the layers and weaken the structure.

Figure 2.4. Diagrams of a discrete/layered microstructure and a continuously graded microstructure [51]

Delamination between layers can happen in sandwich structures, where each part has a foam core and two high-density face sheets sandwiching the foam core. The foam core has low density with stiffness much lower than the face sheets. When the structure is loaded, there are incompatibilities between the core and face deformations, leading to stress concentration at the interfaces between the core and face sheets. When the concentrated strain energy at the interface is released, the interfaces are delaminated [93].

Mahfuz *et al.* [94] performed a buckling test for three types of sandwich structures with the same face sheets but different foam core densities with embedded delamination at the interface. The results showed that the structure with the highest core density (the closest density to the face sheet density) had minimal delamination propagation. This showed that less abrupt density transition will lessen the likelihood of delamination. Thus, it is preferred that the scaffold functional gradients avoid abrupt structural changes to prevent delamination.

2.2. Computer-Assisted FGS

In order to build better TE scaffolds for the human body, the major challenge is the customization of the scaffold's micro- and macro-architecture so that its biological and mechanical properties could mimic the native tissue [25, 95]. Additionally, the external

geometry of the scaffold has to anchor and fit well into the defect site as it will determine the stress distribution at the organ-implant interface and also the surrounding tissue (anatomical requirement) [21-22]. These challenges pose problems to the scaffold design stage, resulting in it being tedious and complicated. Several researchers have proposed the use of computer-aided tissue engineering to ease the design and control of the scaffold macro- and microstructures [21-22, 34-35]. In general, such CAD software involves a collection of repeatable structural units that can be selected and stacked to form a scaffold, thus customized scaffolds are more easily and readily reproducible.

The general approach of computer-aided tissue engineering as suggested by these researchers can be summarized as follows [11, 35]:

1. Acquiring the spatial information of the defect site using computed tomography (CT) scan or magnetic resonance imaging (MRI) to obtain the enveloping shape of the defect for reconstruction.
2. Opening a library of various shapes of structural units (called ‘unit cells’) for the scaffold internal architecture and selecting the appropriate unit cell.
3. Stacking up the cellular units to construct a block with larger dimensions than the defect size.
4. Performing Boolean operation between the spatial information acquired from the defect image and the stacked cellular units, resulting in a scaffold having desired internal architecture with enveloping shape of the tissue defect.

Several notable works on computer-aided tissue engineering including the works by Hollister *et al.* [35], Chua *et al.* [21-22]; Cheah *et al.* [36], Wettergreen *et al.* [37-38] and Naing *et al.* [11] are presented in this section:

1. Hollister *et al.* [35] presented an image-based design (IBD) approach to design TE scaffolds using voxel, which is the smallest unit volume of a computer image. The approach worked by setting the voxel within a unit cube to be either 0 for no material (hollow/void) or 1 for material (solid). Depending on the voxel setting, both regular and random pores within the unit cube can be created. The unit cube was repeated afterwards. Two important parameters of IBD were the scaffold porosity and stiffness,

where homogenization theory was used to calculate the scaffold stiffness based on the scaffold architectural design [96]. The benefit of the IBD is its ability to generate a certain value of scaffold density based on a given tissue type and the required stiffness [97]. One disadvantage of this method is that Boolean operation between the designed scaffold and the anatomic defect image must be done in the form of voxel datasets and both datasets must have the same resolution [96]. This means that the operation can only be carried out using specialized software that can process voxel datasets and the files cannot be readily viewed or manipulated using commercial CAD software like Pro/Engineer or SolidWorks.

2. Wettergreen *et al.* [38] created a library of structural units, called unit blocks, which exhibited symmetry along the three orthogonal axes and thus were considered as orthotropic. Two types of unit blocks were created. The first type was space filling solid structures such as spheres and cubes with voids superimposed on them by Boolean operation to be created as pores. The second type was a wireframe approximation of basic polyhedral shapes. To assist the mating between two different types of unit block, a common mating feature of a torus shape was accommodated into each unit block. The system was used to construct scaffold replacement for the entire vertebral body [37]. The advantage of the created library is that each type of unit block had different Young's modulus. One can adjust the modulus by adjusting the porosity of a selected unit block or use different type of unit block while keeping the porosity the same [38]. This provides the user with wider range of porosity-modulus combination to better suit the target tissue. The creation of FGS was made possible due to the common mating feature to assemble two dissimilar unit blocks. However, the generation of radial FGS, especially for long bone regeneration, was not yet considered which makes it an interesting future research to work on.
3. Chua *et al.* [21-22] investigated suitable polyhedral shapes for use in scaffold modeling in order to create a parametric library of scaffold structures. Cheah *et al.* [36] continued the work by developing a novel algorithm to create internal architecture of TE scaffolds by assembling open polyhedral cells into a scaffold

structure. With open polyhedral structures as the unit cells, it can ensure that the scaffolds have 100% pore interconnection for smooth nutrient and waste transport, as well as cell migration. This work was further refined by Naing *et al.* [11] and the system is called Computer-Aided System for Tissue Scaffolds (CASTS). Thirteen configurations of polyhedral unit cells were available to be assembled into customized scaffolds with the incorporation of calculation of important scaffold parameters, such as pore size, porosity and surface area-to-volume ratio. Having thirteen polyhedral configurations could provide a wide range of scaffold selections with various combinations of pore size, porosity, and mechanical properties. Additionally, the availability of the option to incorporate nodes at the edges of the struts and/or additional struts reinforcement can improve the mechanical properties of the scaffolds. However, the incorporation of mechanical properties such as stiffness and strength related to the scaffold porosity and structure has not been done yet on this system. This system has not been complemented with the ability to generate FGS, hence, it can only generate scaffolds with uniform microstructure throughout. These aspects have been considered for future development of CASTS system.

4. Lian *et al.* [98] proposed a computer-aided modeling approach to design fiber structures with radial porosity gradient using 3D concentric microstructures. The 3D concentric microstructures were composed of a stack of 2D concentric structures, which were generated based on the unified mathematical model. In the model a circle was evenly divided into m number of segments, which corresponded with m number of points. As a start, one point was linked using a line into a certain neighboring point. This step was repeated for all the points on the circle, resulting in a 2D concentric structure (Figure 2.5 (a)). A 2D concentric structure was connected to the neighboring structure by connecting each point on one structure to a point on another structure that had the shortest distance. This process resulted in a connected stack of 2D structures, forming a 3D structure (Figure 2.5 (b)). To obtain concentric chitosan-collagen fibers, a mold was required so that the fibers can be wrapped around it to form the final product. A drawback of this system is that the design of the concentric structures and the design of the mold involve two different softwares. The design of the concentric

structures was carried out in special software using JAVA language, while the mold design was done in separate CAD software for RP fabrication. The mold design is not automated, hence, it creates additional manual step after the concentric structure design. Furthermore, the manual wrapping of the fibers around the mold depends much on the user skill, hence less reproducible.

Figure 2.5. (a) A sample of 2D concentric structures and (b) a 3D concentric structure obtained from joining two 2D concentric structures [98]

5. Cai and Xi [99] presented bone scaffold modeling based on hexahedral mesh refinement of the bone defect profile obtained from CT/MRI. The scaffold pore was represented by a sphere inside a unit cube. When the unit cube was deformed inside the meshed bone defect profile, the sphere inside the unit cube followed the deformation according to the unit cube deformed shape. The mesh can be refined when and where smaller pores were needed, allowing the scaffold to achieve non-uniform pore size distribution mimicking the target bone tissue (refer to Figure 2.6). As a result, this system offers good control over the pore size and pore distribution across the scaffold. The limitation of this system is the absence of the porosity calculation, which is important for cell attachment and proliferation and critically contributes to the mechanical properties of the load-bearing bone scaffold. Hence, porosity-mechanical properties relationship is an important feature that should be added into this system.

Figure 2.6. Bone scaffold modeling based on hexahedral mesh refinement [99]

2.3. Biomaterials

Other than the cells themselves, a suitable biomaterial is an essential component in tissue engineering construct. The material should possess biocompatibility, suitable mechanical properties, be sterilizable, and induce cellular interaction and tissue development. Besides the mentioned properties, the biomaterial should also be biodegradable, i.e. it degrades over time, allows the healthy tissue to replace the defect, as well as, possesses non-toxic degradation byproducts, which would not yield ill effect to the surrounding tissue and the body as a whole [2].

The materials commonly used for building tissue scaffolds include [8]:

1. Biopolymers

Generally biopolymers are classified into two categories:

- Natural polymers, e.g. proteins and polysaccharides. Some examples of fibrous protein are collagen and silk. Collagen is a major natural extra cellular matrix component, especially used in soft tissue repair. However, there are concerns about its poor mechanical properties and less controlled degradation rate. As for silkworm silk, it has a very slow degradation rate and there is also a concern over cytotoxicity.

- Synthetic polymers, e.g. poly-L-lactic acid (PLLA), polyglycolic acid (PGA), poly-lactic acid - co - glycolic acid (PLGA), polycaprolactone (PCL), polyetheretherketone (PEEK) and polyvinyl alcohol (PVA). PGA degrades through hydrolysis of ester bonds. It is relatively hydrophilic and degrades rapidly *in vivo*, while PCL degrades at a much slower rate, making it attractive for long term implants. PEEK, a bioinert polymer, has a relatively high melting temperature, T_m , and glass transition temperature, T_g , making it suitable and stable to be processed at elevated temperature. It can be sterilized by steam autoclave or radiation without experiencing significant degradation in its properties.

Natural biopolymers are considered more advantageous for TE scaffolds and favored by the cells as they are usually parts of natural ECM and provide specific cellular interactions, such as promoting cellular adhesion, differentiation, and migration. However, often they have poor mechanical properties and cannot be processed at elevated temperature. Synthetic biopolymers provide greater versatility in designing scaffolds with controlled properties, such as macrostructure, mechanical properties, and degradation rate. They are also more versatile in terms of their processability [79]. Although they do not provide receptor-binding ligands for cells [100], they can be coated using suitable protein to enhance their biocompatibility, e.g. collagen [101].

2. Bioceramics, e.g. porous bioactive glasses and calcium biophosphates.

Most frequently used materials from the calcium biophosphates class include tricalcium phosphate (TCP) and hydroxyapatite (HA). These materials are considered osteoconductive and osteoinductive [102]. Osteoconductive means that the surface properties of the material support osteoblastic cell adhesion, growth, and differentiation, while osteoinductive implies that the material can induce bone formation. However, the drawbacks of these materials are that they are difficult to process into a highly porous scaffold as they are mechanically brittle [102]. One way to overcome the disadvantages is to mix the material with other material, forming a composite material [103].

3. Biocomposites, which are a combination of two or more constituent materials previously mentioned e.g. biopolymers and bioceramics, such as PLLA-HA, PLGA-HA [104], PEEK-HA [103, 105], and PVA-HA [106-107]. As mentioned previously,

HA is difficult to process as it has poor mechanical properties, making it very brittle. However, its mechanical property can be enhanced if it is mixed with other material, such as PCL or PVA to make it suitable for TE applications. These biocomposites become easier to shape and are mechanically sound because the biopolymer acts as a binder for HA particles, making the scaffold less brittle [102, 108]. They promote more cell adhesion and colonization than those of pure polymer as HA improves the hydrophilicity of the biopolymers [109].

2.4. Methods for Building Functionally Graded Scaffolds

TE scaffolds can be fabricated in a variety of ways, which are broadly classified as conventional and advanced processing methods [8, 14-15].

2.4.1. Conventional Methods

Scaffold fabrication techniques such as fiber bonding, solvent casting/particulate leaching, gas foaming, phase separation and freeze drying are generally classified as conventional methods [4, 8, 15-16, 110-111]. These techniques rely heavily on user skills and experience; therefore the microstructure of the scaffolds fabricated is usually not consistent. The use of toxic solvents, such as chloroform, and porogen in these processes limits the thickness of the scaffolds to facilitate the removal of the solvents and porogen. Moreover, the solvents residue in the scaffolds can be toxic to the cells. The scaffolds fabricated by these techniques are also limited to simple geometries, which may not fit well into the defect shape of the tissue/organ [8, 16].

Nevertheless, some of these techniques have been shown to be able to fabricate scaffolds with porosity and/or pore size gradients. The most promising of such techniques are discussed in the following sections:

1. Solvent casting/particulate leaching

This method involves stacking powder mixture layers containing different volume fractions and/or particle sizes of volatile sintering additives such as ionic salts. Werner

et al. [27] used a technique called multiple tape casting to produce hydroxyapatite (HA) scaffolds with porosity and pore size gradients (refer to Figure 2.7). After the multilayer scaffold was prepared, the polybutylmethacrylate (PBMA) particles that acted as the porogens were burnt out and sintered, resulting in pore sizes ranging from 70 μm to 200 μm . Schwarz and Eppler [112] fabricated polyglycolide scaffolds resembling natural bone with a cortical (compact) and a cancellous (porous) part using sodium chloride crystals as porogens. The salt crystals were then washed out with water, leaving interconnected pores with size in the micrometer range. The sizes of the pores were controlled by the salt particle size while the porosity was controlled by the proportion of the salt crystals. Vaz *et al.* [113] incorporated polyvinyl polyacrylate (PVPA) to create pores in multiple slip casting technique (for illustration, refer to Figure 2.8) and fabricated HA bone implant with porosity gradient.

Figure 2.7. Multiple tape casting process [27]

Figure 2.8. Slip casting process [114]

Both multiple tape casting and slip casting make use of porogens to form the pores of the fabricated scaffolds. The scaffold pore size is defined by the porogen particle size, while the porosity is defined by the amount percentage of the porogen. An advantage of this method is that the pore size and porosity are independent of each other, making the design of desired pore size and porosity simpler. However, this process has limited control over the pore interconnectivity, in which one porogen particle must come into contact with the surrounding porogen particles to form desired pore interconnection. Another down side is that it is difficult for this process to form complex shapes such as human vertebrae.

2. Impregnating cellulose sponges into HA slurries

Tampieri *et al.* [28] made HA slurries of dissimilar characteristics using HA powders with different degrees of crystallinity. Two types of porous body were obtained from soaking cellulose sponges into the two different slurries (slurry L from 20% crystalline powder, slurry H from 80% crystalline powder). First, the sponge was completely impregnated in slurry H. Subsequently the same sponge was partially impregnated in slurry L. Afterwards, the sponge was left to decant and air-dried, and finally it was sintered. The resulting scaffold possessed a low-porosity region, resulting from the additional partial impregnation in slurry L, and a more porous region, resulting from the first complete impregnation in slurry H (refer to Figure 2.9).

The overall porosity of the sample was 67% while the individual porosities of low and high porosity regions were not reported.

Figure 2.9. (a) High porosity and (b) low porosity cellulosic sponges [28]

The properties of the scaffold fabricated using this impregnation method are highly dependent on the properties of the sponge used, including the sponge pore size, porosity, and pore interconnections. Hence, this process is prone to batch-to-batch variations and limited to relatively simple shapes. The initial porosity of the sponge and how the sponge impregnation changed the final scaffold porosity were also not mentioned.

3. Self-foaming

Zeschky *et al* [115] obtained porosity gradient by setting lower foaming temperature to control the bubble nucleation, growth, and bubble rise. In Figure 2.10 (a), it is noticed that when the polymer was heated at 270°C the curing process was fast, the viscosity of the polymer increased rapidly and prevented the rise of the gas bubbles making the porosity constant throughout the foam. However, when the foaming temperature was set below 260°C, i.e. at 220°C as shown in Figure 2.10 (b), the curing process slowed down. The lower viscosity made the bubble rise. The longer the time, the bigger the bubble grew while the viscosity increased. It resulted in smaller bubbles at the top part of the foam while the bigger bubbles were at the bottom part of the foam, which can be seen Figure 2.10 (c).

Figure 2.10. Illustration of the foaming process stages (a) at 270°C (homogeneous porosity) and (b) at 220°C (gradient porosity) (c) Structure with gradient porosity obtained from self-foaming process [115]

By controlling the temperature and time duration of the foaming process, the foam pore size can in turn be controlled. However, there is lack of control over the pore distribution and arrangement, for example: when it is desired that the foam has either linear or radial pore size/porosity. Complex foam shapes are difficult to achieve too.

4. Centrifugation method

Harley *et al.* [116] fabricated collagen-based, porous tubular scaffolds to facilitate the study of myofibroblast migration during peripheral nerve regeneration. The collagen-glycosaminoglycan (CG) suspension in acetic acid was spun in a cylindrical mold about its longitudinal axis to separate the components (liquid and solid phases) and rapidly frozen to lock the component separation. Finally the frozen solvent phase was

removed via sublimation. The resulting scaffolds possessed radially aligned pore structure with pore size ($<5\text{--}20\text{ }\mu\text{m}$) and porosity gradient along the radius, where the outer portion of the tube had smaller pores compared to the inner portion. Oh *et al.* [117] obtained PCL scaffold with pore size ($\sim 88\text{--}405\text{ }\mu\text{m}$) and porosity ($\sim 80\%\text{--}94\%$) gradients using centrifugation method followed by fiber bonding by heat treatment. The scaffold was fabricated to investigate the interaction between cells and scaffold with varying pore size.

Depending on the axis of rotation, the centrifugation method is able to fabricate pore size/porosity gradient in either linear or radial direction. Nevertheless, there is lack of control over pore arrangement and distribution, as well as, the difficulties in producing scaffolds with complex shapes. There is also the need for solvents, which may be toxic to the cells if not removed properly, as the materials need to be in suspension for this process to be carried out.

5. Foam replication technique

As a variation of impregnation technique, Bretcanu *et al.* [118] presented the fabrication of Bioglass scaffolds with porosity gradient for bone application. Continuous and stepwise gradients were fabricated using preformed polyurethane (PU) foams as sacrificial templates. PU foams were preformed using aluminum foil molds. Depending on the mold shapes, different values of porosity gradient were obtained, e.g. a rectangular foam was put into a trapezoidal mold to get a scaffold with continuous porosity gradient (refer to Figure 2.11). Stepwise gradient was achieved by putting two sponges with different porosity in the same mold. The foams were preformed by compressing some parts of the foams using the mold at 200°C for 30 minutes. The preformed PU foams were then immersed in Bioglass slurry and heat treated in a furnace up to 1100°C to sinter the Bioglass and burn out the PU foams, resulting in a porosity graded Bioglass scaffold.

Figure 2.11. (a) PU foam before preforming, (b) PU foam inside a mold, and (c) preformed PU foam with continuous porosity gradient [118]

This technique provides simplicity in fabricating scaffolds with pore size and porosity gradient. The local pore size at a certain location on the foam can be calculated according to a linear relation, where the pore size decreases with increasing pore distance from the foam side with larger porosity. However, how the mold shape affects the porosity distribution throughout the scaffold was not mentioned. Also, this technique involves multiple steps (from foam cutting and preparation, mold preparation, foam preforming, foam impregnation, and finally heat treatment) and is highly dependent on user skill, making the process prone to batch-to-batch variations. Additionally, like the sponge impregnation technique, the properties of the scaffold fabricated using this method are highly dependent on the properties of the foam used, including the sponge pore size, porosity, and pore interconnections. This process is also limited to relatively simple mold shapes.

From the above review, it can be summarized that the techniques mentioned have inherent limitations, such as poor control over pore size, porosity, pore interconnectivity, and the spatial distributions of the pores [8, 15-16]. These techniques are highly process dependent rather than design dependent, therefore the resulting structures are not readily reproducible [24]. Thus, conventional techniques are not considered suitable for fabricating complex structures such as patient-specific FGS, which needs a high degree of

control over the scaffolds' macro- and microarchitectures. A technique with better control over scaffold parameters such as pore size, porosity, etc. is needed to produce customized scaffolds. In this case, rapid prototyping (RP) or solid freeform fabrication (SFF) is seen as a viable alternative technique to produce complex scaffolds with controllable parameters, such as a patient-specific FGS [8, 14-15, 119].

2.4.2. *Advanced Methods*

RP is a technique for manufacturing physical models from Computer Aided Design (CAD) data, which is categorized as an additive process. In this process, models are constructed layer by layer until the desired three-dimensional object is formed [14-15, 120]. Apart from constructing medical models, RP can also be used to fabricate tissue scaffolds [121]. Due to the nature of its layer-by-layer fabrication principles, RP is deemed to have advantages over conventional techniques in terms of flexibility, accuracy and repeatability in fabricating structures with complex architecture including parts with structural gradients. The more prominent RP processes are namely: Fused Deposition Modeling (FDM) [122-126], 3D Printing (3DP) [119, 127-128], 3D Bioplotting [129-131], ModelMaker II (MMII) [120, 132-134], Stereolithography Apparatus (SLA) [135], and Selective Laser Sintering (SLS) [26, 105-106, 136].

Scaffold fabrication involving the use of RP begins with the patient's data, which is the tissue defect shape obtained from medical imaging techniques like Computer Tomography (CT) scan or Magnetic Resonance Imaging (MRI). These data will be converted into an IGES (Initial Graphics Exchange Specification) file format or other appropriate graphics format by using an imaging software, such as MIMICS [11]. The IGES file format is a neutral file format used to transfer files between different systems. This data will then be processed by using CAD software (e.g. Pro/Engineer) to produce the model of a customized scaffold, which will finally be built using a RP system [129, 137]. Landers *et al.* [129] summarized the general overview of TE, CAD, and RP combination as shown in Figure 2.12.

Figure 2.12. General overview of TE, CAD, and RP combination [129]

The RP processes for TE can be classified into direct and indirect RP fabrication processes [15, 138-139]:

1. Direct RP process

In this method the scaffold is constructed directly by RP without involving the use of a mold. Some examples of direct RP techniques are FDM, 3D Plotting, SLS, and 3DP. There are also literatures reporting scaffold fabrications that make use of direct RP combined with conventional processes, such as salt leaching [12, 20]. Zeltinger *et al.* [12] produced 3D scaffolds by using the TheriForm process, a solvent-based RP process, combined with the leaching of the porogen. PLLA powder was mixed with salt (NaCl), which acted as the porogen. The polymer powder was swelled and dissolved by the solvent binder. The polymer precipitated around the porogen after the solvent evaporated. The resulted scaffold was porous after the porogen leaching. Likewise, Sherwood *et al.* [20] fabricated an osteochondral scaffold using Theriform process. The porosity was varied by varying the amount of salt (NaCl), which acted as porogen, spatially across the scaffold. The porogen was then leached with water.

2. Indirect RP process

Unlike direct RP processes, indirect RP processes build a master pattern of the scaffold and then use it to produce a sacrificial mold to fabricate the final scaffolds.

Indirect techniques are usually used to conserve original material properties e.g. natural biopolymers, as the process does not involve high temperature [138]. By using this method, a wider range of materials can be used to make the final scaffold. However, organic solvents are still involved in indirect RP processes, and the scaffold resolution depends solely on the mold resolution. The techniques under this category are FDM, MMII, and SLA [15].

Some RP techniques that are able to fabricate complex structures such as FGS are discussed as follows:

1. Theriform

Sherwood *et al.* [20] fabricated an osteochondral scaffold with porosity and material gradient by using Theriform three-dimensional printing process, which is a powder-based RP that makes use of a binder to join the powder particles to form a three-dimensional object. The porosity was varied by changing the amount of the porogen (in this case, NaCl) spatially across the scaffold. The porogens were then leached using water. The upper region was the cartilage region and was made of PLGA/PLA, while the lower region was bone region and was made of PLGA/tri-calcium phosphate (PLGA/TCP) to promote bone ingrowth. The difference in the porosity of the cartilage (90%) and bone region (55%) was meant to prevent the chondrocytes seeded in the cartilage region from migrating towards the bone region of the scaffold and also to give the bone section more mechanical strength. A larger pore size was used in the bone region ($>125\text{ }\mu\text{m}$) to facilitate the mineralized bone ingrowth. Between the cartilage and bone region, there existed a gradual transition of the material and porosity values (85%, 75%, and 65%) to overcome delamination at the interface of the two regions due to shrinkage (refer to Fig. 2.13).

Figure 2.13. The variation of porosity and pore size with respect to the position in the scaffold fabricated using Theriform [20]

In this process, osteochondral scaffolds with material and porosity gradients have been fabricated. The gradients were a success as they not only fulfilled the different cell requirements for different regions, i.e. bone and cartilage regions, but also helped in preventing delamination between the two regions due to different shrinkage coefficients. The elastic modulus achieved for the bone section (83-200 MPa) was in the range of native human cancellous bone. The staggered channel design and the porosity difference achieved the objective of guiding the chondrocytes to preferentially attach to the cartilage region of the scaffold. However, a confirmation should have been done to seed osteoblast onto the bone region of the scaffold to ensure that not only the chondrocytes attach to the correct region but also the osteoblast preferentially attach to the intended bone region. Another concern in this technique was the use of toxic solvent (chloroform) in the fabrication process, which caused the need for additional post-processing to completely remove the residual solvent before performing cell seeding on the scaffolds.

2. Fused Deposition Modeling (FDM)

FDM is a solid based RP system (schematic diagram shown in Figure 2.14), which takes in filaments as the input material. The filament is fed into an extrusion head, heated to a semi-liquid state, and then extruded through a fine nozzle in thin layers. The head moves on x-y plane according to the tool path to create a desired cross-sectional layer. Upon extrusion and deposition, the material solidifies and forms part of the 3D object. The process is repeated layer by layer until the object is complete [120].

Figure 2.14. A schematic diagram of FDM process and FDM parameters, including slice thickness (ST), road width (RW), and raster gap (RG) [124]

Zein *et al.* [123] used FDM to produce novel honeycomb-like PCL scaffolds with fully interconnected pores, controllable porosity, pore size, and analyze their mechanical properties. The achievable scaffold porosity range was 48-77%, while the compressive stiffness and yield strength were 4-77 MPa and 0.4-3.6 MPa, respectively. The scaffold's compressive stiffness and yield strength were found to decrease rapidly as the porosity increased. Looking from the achievable scaffold stiffness range, these scaffolds could be used for cancellous bone regeneration.

Kalita *et al.* [126] used FDM to build several concentric cylinders with different pore sizes radially from segment to segment. The material used was polypropylene/tricalcium phosphate (PP/TCP). The resulting scaffold had different discrete rings of constant porosity in the radial direction, and was directed for bone implants especially for cancellous bone grafts. Gradient porosity, pore size and pore shape were fabricated by varying the raster gaps and lay-down patterns for each concentric ring. The scaffold shown in Figure 2.15 was fabricated using constant lay-down pattern of 0-60-120 with varying the raster gap (2.03 mm at the center, 1.27 mm

in the middle section and 0.51 mm at the outer ring) and had decreasing pore size and porosity towards the outer section.

Figure 2.15. Scaffolds with graded structure in radial direction fabricated by FDM [126]

FDM, like other RP systems, has inherent advantages including the ability to fabricate parts as what is designed in the CAD software, with the example of the FGS shown in Figure 2.15. Unfortunately, sectional views of the mentioned FGS were not provided in the article to confirm pore interconnectivity at the interfaces between two concentric rings. It is suspected that there are pore discontinuities at the interface, which are due to the scaffold design and can obstruct cells infiltration, as well as nutrient and waste diffusion towards the center of the scaffold.

3. 3D Fiber deposition (3DF)

Woodfield *et al.* [24, 31] fabricated a graded structure for articular cartilage application using 3DF by varying the fiber spacing (refer to Figure 2.16). 3DF has the same fabrication principles as FDM with a difference in the input material. The input material for 3DF is in pellet form rather than filaments in FDM. The fiber deposition method produced scaffolds with 100% interconnected pores, which was confirmed by the SEM images shown in Figure 2.16. The resulting scaffolds had pore size range of 150-1650 μm in the x-y plane and 170-195 μm in the z direction, which was due to the fiber diameter. The scaffold porosity ranged between 59-87%, which is considered high and desirable for cell proliferation. The study was followed by *in vitro* cell

seeding. Pore size gradient promoted homogeneous cell distribution imitating those of natural bovine articular cartilage, as confirmed by in vitro experiments carried out on the scaffolds. By controlling the cells distribution it was found that the zonal ECM production was affected to a certain extent to conform to those of native bovine articular cartilage, which in turn will influence the biomechanical function of the cartilage [32, 140].

Figure 2.16. Scaffolds with fabricated by 3DF, (a-c) with uniform pore sizes and (d-f) scaffolds with pore size gradient [32]

4. Multinozzle Low-temperature Deposition and Manufacturing (M-LDM)

Liu *et al.* [141] developed a solvent-based deposition technique called Low-temperature Deposition and Manufacturing (LDM). The input material for this system is solvent-dissolved polymer, which is extruded through a nozzle onto a platform at temperature below 0°C. The resulting parts are in frozen state and need to be freeze-dried to remove the solvents and to create micro-pores in the extruded filaments. The LDM system was improved into M-LDM [142] to allow multiple nozzles to extrude more than one type of polymer, resulting in scaffolds with both material and structural gradients (refer to Figure 2.17).

Figure 2.17. Schematic diagram of M-LDM process [142]

M-LDM allows scaffolds with both material and structural gradient to be fabricated, which could facilitate the regeneration of multiple tissues using one scaffold, e.g. bone-cartilage (osteocondral) scaffolds. Moreover, as the process is carried out at low temperature, the processing of temperature sensitive natural polymers, such as collagen, is made possible. As such, it extends the range of available raw material that can be processed using this technique. One of the limitations includes the relatively longer lead time to achieve a final scaffold as the process involves multiple steps, such as dissolving the polymer, selecting suitable nozzle type for the polymer used, the fabrication process itself, and freeze drying. The incorporation of solvent into the process could be toxic to the seeded cells if the solvent is not properly removed. The resolution of the resulted scaffold is $\sim 500 \mu\text{m}$ [141], which is considered low. This is because the deposited polymer lines are larger than the nozzle diameter due to the fact that the polymer solution did not freeze directly upon extrusion [141]. More research must be done to find the suitable deposition temperature in order to freeze the polymer directly upon deposition and reduce the dimensional error.

5. Selective Laser Sintering (SLS)

SLS (depicted in Figure 2.18) is a powder based RP system that makes use of a CO₂ laser beam to fabricate 3D solid objects [120]. The SLS fabrication starts with powder deposition onto the part bed by the roller. The cross-sectional area of the part is selectively scanned by the laser on the powder layer. The part bed lowers down one layer thickness and a new layer of powder is deposited on top of the previous layer. The process is repeated until the part is complete [105].

Figure 2.18. A schematic diagram of SLS [120]

As SLS involves fusing the powder by laser power, one challenge in using SLS to fabricate scaffolds from biomaterials directly is in investigating how the laser affects the properties of the biomaterials. As such, the biomaterials have to be carefully selected so that they do not degrade during the sintering process. Tan *et al.* [136] varied the three SLS parameters, namely the laser power, the part bed temperature, and the laser scan speed, for sintering biomaterials such as PEEK, PVA, PCL, PLLA, and HA to determine the optimal sets of parameters for each biomaterial. Besides the mentioned parameters, particle size of the powder also influences the accuracy and surface finish of the final scaffolds. Williams *et al.* [26] suggested that the optimal biomaterial powder size should be in the range of 10-100 μm . The porous nature and surface roughness of SLS sintered powder could be beneficial for cell attachment. On the other hand, the wide range of materials that the SLS can process [120] makes this technique suitable for TE scaffold fabrication. Some limitations include the low

resolution of 300-400 μm and the high temperature involved in the process, which hinders the processing of temperature sensitive materials.

As a summary, a preferred fabrication technique for TE scaffolds should be able to process a selected biomaterial without degrading it, achieve the intended macro- and microarchitecture, achieve the desired mechanical properties and free from the use of toxic solvents. Judging from these criteria, FDM and SLS can be considered to be the most promising in terms of fabricating scaffolds for bone applications. Although high temperature is involved in both techniques, in general the materials used for bone scaffolds are synthetic polymers, which can stand to be processed in elevated temperature [79]. Synthetic polymers are preferred as they provide better mechanical properties for load bearing scaffolds compared to natural polymers. An advantage of SLS over FDM is the absence of support structures for overhanging features in the SLS process. Hence, scaffold cleaning/post-processing is simpler for SLS fabricated scaffolds as the loose powder that supports the overhanging features can just be blown away from the scaffold using compressed air [120].

2.5. Summary

Current computer-aided TE systems have greatly eased the design of uniformly structured TE scaffolds with complex requirements, i.e. biological, mechanical and structural needs. Various types of scaffold structure library have been developed, each having unique cellular unit structures, such as voxel-based, solid-based and wireframe-polyhedral- based unit cells. Scaffolds with controllable and repeatable microstructures can be fabricated based on user's input and requirements. Efforts in the direction of FGS fabrication have been made. For example: the scaffold library constructed by Wettergreen *et al.* [38], in which a common connector feature of a torus shape was added at every sides/facets of each cellular unit. This feature acted as a bridge to allow a smooth change between two different cellular units. Other examples include the works by Lian *et al.* [98] and Cai and Xi [99], which allow the generation of radial FGS for bone application.

Another critical aspect in the FGS design includes the establishment of the relations among the scaffold biological properties (e.g. pore size and porosity required by target tissue), mechanical properties (e.g. stiffness and strength), and the scaffold physical parameters (e.g. strut length and strut diameter). The nature of the gradient specific to target tissue, i.e. linear or radial FGS, must be taken into consideration, as well. The works of Hollister *et al.* [35, 96-97] and Wettergreen *et al.* [37-38] have involved the biological and mechanical consideration to a certain extent into their CAD systems. However, the topic on the type of the gradient, especially the radial gradient for long bone regeneration, has not been investigated yet.

Preferably, after the relationships between scaffolds requirements, i.e. biological, mechanical and anatomical requirements and the scaffold physical characteristics have been well established, such relationships can be incorporated into the CAD systems in order to help tissue engineers design a customized FGS that fulfill the requirements mentioned. This would be the likely direction of CAD aided TE systems development in the near future. The application of medical imaging systems (e.g. CT scan and MRI) and CAD systems have pretty much dealt with the problem of matching anatomical requirements of the TE scaffolds. Hence, more focus will be given to the biological and mechanical aspects, including the nature of the gradient (linear/radial gradient).

The next task to be carried out would be to establish the relationships of both mechanical and biological requirements and the scaffold structural parameters such as pore size and porosity. Other scaffold properties such as surface roughness and surface chemistry are as important. However, these properties are fabrication process or material dependent and cannot be controlled through the CAD scaffold design. Hence, the selection of the scaffold material and fabrication means should also be given a thoughtful consideration for the success of the scaffolds. Last but not least, ways to incorporate linear and radial gradient into the CAD systems should also be explored.

The likely major controlling factor would be the functions of the tissues and cells at the local and cellular level. Thereafter, conflicting requirements, i.e. high porosity is desired

for cell proliferation (biological need) but detrimental for scaffold strength and stiffness (mechanical need), can be resolved by determining the optimized scaffold structure such as pore size, porosity and the gradient to better suit both biological and mechanical requirements. The difficult challenge in matching these relationships to the physical characteristics of the TE scaffolds can be met by understanding the specific functional requirements of the tissues and organs themselves. Mimicking the physical environment can be a first step and further studies and experiments will be needed to understand the cellular behavior and response to these physical parameters. While some information is available in one form or another in literature, there is a need to assemble these bits of information in a meaningful way such that a good model can be established upon which a suitable computer-aided tissue engineering system can be developed.

Chapter 3 – METHODOLOGIES OF SCAFFOLD DESIGN AND FABRICATION

As discussed in Chapter 2, designing customizable scaffolds with desired internal and external architecture to satisfy biological, mechanical, and anatomical requirements is tedious and time-consuming. Therefore, a system, which has a wide range of designs to choose from and can generate and customize the scaffold according to patients' specific needs, is required. This is where the Computer Aided System for Tissue Scaffolds (CASTS), which was built in previous works by Naing [11], contributes as a platform for building an automatic FGS design system to meeting these requirements.

This chapter presents the scaffold design and fabrication stage of the overall system, where CASTS system, the base for the current work, and Selective Laser Sintering (SLS), the selected scaffold fabrication means, will be discussed in more detail. The biomaterials used in this work, PCL and PCL/HA, will also be presented.

3.1. Computer Aided System for Tissue Scaffolds (CASTS)

3.1.1. Modules of CASTS

The platform of CASTS is Pro/ENGINEER, a three-dimensional Computer Aided Design/Computer Aided Manufacturing (CAD/CAM) system. CASTS possesses three modules, namely the input module, the designer's toolbox, and the output module.

3.1.1.1. Input Module

Besides the standard hardware and software for computer systems, CASTS requires imaging software such as MIMICS (Materialise's Interactive Medical Image Control System) for its input module. This imaging software is for converting raw patient data from Magnetic Resonance Imaging (MRI) or Computer Tomography (CT) scan to surface

files of IGES (Initial Graphics Exchange Specification) format, which is a neutral data format for file transfer to dissimilar system.

3.1.1.2. Designer's Toolbox

The designer's toolbox is to provide CASTS user with a variety of useful tools to customize the scaffold according to patient's specific needs. The tools include:

1. A wide-ranging parametric library, where the user can select a basic cellular unit/packing configuration, which will be repeated to form a scaffold block.
2. Sizing routines, where the user can specify the dimension of each cellular unit and also the overall dimension of the scaffold block in the x, y, and z directions.
3. Automated algorithm to link the choices/values input by the user (basic cellular unit and sizing routines) and the CAD file of the scaffold.
4. Scaffold internal architecture data to allow the user to obtain critical information on the scaffold generated, including porosity, pore size, and surface-to-volume ratio. These values are calculated based on the dimensions input by the user in sizing routines.

3.1.1.3. Output Module

The output of the designer's tool module is a scaffold block in the PRT (part) file format. To customize the scaffold to patient's specific shape, the surface files of IGES format from the input module is then used. After the customization is done, the part file must be converted into the STL file format for scaffold fabrication on the SLS. The STL file format is basically a series of triangular facets, each of which is represented by 3 vertices and a unit normal vector [120].

The interactions among the modules are depicted in Figure 3.1.

Figure 3.1. Modules of CASTS

3.1.2. Polyhedral Unit Cells

The CASTS system consists of polyhedral unit cells that can be assembled together to form a scaffold block. The polyhedra used in CASTS are convex polyhedra, whose shapes are relatively simple so that they can be easily modeled in CAD environment. The assembly process is done by repeating the unit cells and mating their faces with the faces of their adjacent unit cells. Some of the polyhedral unit cells with the resulted scaffold blocks are shown in Figure 3.2. These scaffold blocks are created using the current CASTS system, whose scaffolds are limited to having uniform porosity throughout. In

this research, developments on the CASTS system have been made in order to automate the design of linear/radial FGS, as well as, to incorporate relations between scaffolds biological and mechanical properties. The details on the developments made are discussed in Chapter 4.

Figure 3.2. Six of the polyhedral unit cells with the resulted scaffold blocks obtained from combining the unit cells

3.1.3. Designing Scaffolds using CASTS

The steps to design a scaffold using CASTS (in Pro/ENGINEER environment) are as follows:

1. Firstly, an IGES file of a desired shape, for example a cylindrical disk for cell seeding in 24-well plates (refer to Figure 3.3 (a)), is prepared.
2. A unit cell configuration is selected out of 13 available configurations in CASTS (refer to Appendix page 178).
3. The dimensions of the unit cell and also the overall scaffold dimension in X, Y, and Z direction are specified.
4. A scaffold block is generated, having architecture and dimensions according to the user input in steps 2 and 3 (refer to Figure 3.3 (b)).

5. The cylindrical disk of IGES file format from step 1 is appended within the scaffold block for a Boolean operation to take place (refer to Figure 3.3 (c)).
6. A Boolean operation is done between the scaffold block and the cylindrical disk to produce a scaffold with the shape of a cylindrical disk (refer to Figure 3.3 (d)).

Figure 3.3. Steps to obtain a customized scaffold

After completing the abovementioned steps, the resulted scaffold of PRT file format can then be converted into an STL file for fabrication. The screen prints of scaffold design process using CASTS associated with Figure 3.3 are attached in Appendix page 179-180.

3.2. Selective Laser Sintering (SLS)

SLS is a powder-based RP system that creates three-dimensional object layer by layer from CAD data of STL file format. The SLS model used in this research is the Sinterstation 2500. The main components of the system are a build chamber and a workstation. The build chamber consists of two powder cartridges on either side of the part bed, a part bed with variable height, and a carbon dioxide (CO₂) laser beam. The workstation possesses inbuilt software to process the CAD data and slices the part into thin layers.

The CO₂ laser beam selectively fuses the powder together to build solid 3D objects. The laser beam raises the temperature of the powder particle to the point of melting, causing particle bonding, not only among particles in one layer, but also with the previous layer, forming a solid object [120].

The sintered object is porous and has a relatively rough surface depending on the particle size, thus beneficial for TE scaffold application as the surface roughness would allow the cells seeded on it to attach on the scaffold. The surface roughness value of sintered PCL with particle size $\sim 100\text{ }\mu\text{m}$ was found to be $\sim 30\text{ }\mu\text{m}$ in this work (refer to section 6.2.2).

Multiple parts can be built together by grouping them into batches. Therefore, one layer may contain more than one cross-sectional area. The user can design a build layout by placing and orienting the parts that are going to be built together. The SLS process does not need any additional support structures as the unsintered powder particles can act as a support structure.

There are several parameters of SLS that can be varied depending on the powder material used. The three main parameters are the laser power, the laser scan speed, and the part bed temperature. The combination of the laser power and laser scan speed will determine the fusing rate of the powder, thus determining the porosity of the 3D object. The sintering process requires the temperature of the particles to be raised to its glass transition temperature. Another parameter to vary is powder layer thickness, which depends on the size of the powder particle [103, 105, 136].

Briefly, the steps taken to build parts in SLS are as follows:

1. The powder is first loaded into the SLS machine and the STL file is then transferred into the SLS workstation.
2. The build layout is designed by placing and orienting the parts as desired, including setting the SLS parameters required, for example the laser power, laser scan speed, and part bed temperature. This was done in the SLS workstation. The resulted file is called a build package file (BPF).
3. The build process is then started and continues until the part is complete.

After the build is completed, one can collect the solid parts directly from the SLS machine. There is little post-processing required for the completed parts, which is to remove the unsintered powder from the sintered parts. However, for scaffold parts, which

have intricate geometries, the unsintered powder may get trapped within the scaffold. Therefore, more effort and care are needed to remove the trapped powder. One may use compressed air to blow out the unwanted powder, or use a sieve-shaker to remove the trapped powder.

SLS possesses common advantages over conventional techniques, where almost all RP systems omit the use of toxic solvents in the process of making solid objects. Also, RP is able to fabricate predetermined micro- and macro-architecture of the scaffold with good repeatability [14].

Apart from that, as a powder-based RP system, SLS-fabricated scaffolds possess inherent characteristic of having micro pores all over the fabricated structure, which is not only beneficial for bone cells, but also for any other cell types. These micro pores, unique to powder-based RP systems, help cells to attach on the scaffold. Furthermore, SLS is known to be able to produce scaffolds with mechanical properties comparable to cancellous bone [26, 143]. The achieved mechanical properties result from the scaffold structure, which can be designed via CAD system, and the scaffold material. Hence, being a fabrication system that can be used with a wide range of processing materials, scaffolds with optimal mechanical properties for bone regeneration can be researched on using SLS. These advantages, along with the limitations of the SLS process for building TE scaffolds, are summarized in Table 3.1.

Table 3.1. Advantages and limitation of SLS process with regard to TE scaffold applications

Advantages	Limitations
Good part stability	Trapped powder within intricate geometries
Wide range of processing materials	High temperature included, hence may not be suitable for thermally-sensitive materials
No support structures required	Limited laser resolution (300-400 μm for Sinterstation 2500)
Porous structures with some surface roughness obtained	
No toxic binders or solvents used	

Based on these advantages, SLS has been chosen as the fabrication technique for bone TE scaffolds.

3.3. Biomaterials

Two types of biomaterials for bone tissue applications were used in this research, including a biopolymer, polycaprolactone (PCL), and a biocomposite, polycaprolactone/hydroxyapatite (PCL/HA). The selection rationales and details regarding the materials are discussed in sections 3.3.1 and 3.3.2.

3.3.1. Polycaprolactone (PCL)

Polycaprolactone or PCL is a synthetic polymer that has been approved by Food and Drug Administration (FDA) for use in the human body. A semi-crystalline polymer that belongs to polyester family [144], it is biocompatible and degrades *in vivo* by hydrolytic mechanism. The degradation products are non-toxic, which include caprolactone and 6-hydroxyhexanoic acid [145], and are thus taken up by macrophages and degraded intracellularly [146]. Although it has a long degradation time (in the order of two to three

years [147]), it can be compatibly blended with other polymers such as PLA, PLGA or polyhydroxybutyrate (PHB) to tailor the degradation time [148-149]. Furthermore, it has also been shown that PCL scaffold supports cell growth in bone applications [144]. As such, PCL is chosen as the biomaterial used in this work.

PCL powder used in this project was purchased from Solvay Interlox Pte Ltd, UK under the brand name CAPA 6501. The powder is irregular in shape as shown in Figure 3.4 with molecular weight of 50000. The material specifications of PCL are summarized in Table 3.2. The PCL powder in this project is used as received.

Figure 3.4. SEM micrograph of PCL powder

Table 3.2. PCL material specifications [150-151]

Properties	Values
Particle shape	Irregular
Particle size	99% <100 µm
Powder density	1.15 g/cm ³
Glass transition temperature	-60 °C
Melting temperature	58-60 °C

3.3.2. Polycaprolactone/Hydroxyapatite (PCL/HA)

Hydroxyapatite (HA) is a ceramic material whose composition is similar to bone mineral, giving it bioactivity and biocompatibility [76]. However, HA is difficult to process into a highly porous scaffold as they are brittle [102]. Hence, it is usually used to reinforce or fill polymer material for bone substitute, for example PCL [152-153]. The use of HA in a blend with PCL was reported to increase osteoblast differentiation, improve the amount of mineralized matrix [154] and also increase the modulus of the composite scaffolds [143, 152]. For a comparison, the modulus of HA is 114 GPa [155], while PCL is 400 MPa [156].

HA powder was purchased from Plasma Biotals Ltd under the brand name of CAPTAL 'R' with average particle size of 5 μm (refer to Figure 3.5).

Figure 3.5. SEM micrograph of HA powder

PCL and HA powders were mixed using a roller mixer (CZ98174, US Stoneware) at a speed of 200 rpm for 2 hours. HA powder amount was 10 wt% of the final composite weight to obtain the optimum increase in mechanical properties, such as compressive stiffness and strength, based on the work of Wiria *et al.* [143].

In order to check for homogeneity, powder mixtures were observed under the Scanning Electron Microscope (SEM) (JEOL JSM-5600 LV). Figure 3.6 shows two SEM images of the PCL/HA powder mixture, where the red arrows point to some of the HA particles. The HA particles can be differentiated from the PCL particles, owing to its much smaller size ($\sim 5\text{ }\mu\text{m}$) compared to PCL particle size ($\sim 100\text{ }\mu\text{m}$). It was found that the HA particles were evenly distributed among the PCL particles. These mixtures can then be loaded into the SLS as scaffold material.

Figure 3.6. SEM images of PCL/HA mixture

Chapter 4 – FUNCTIONALLY GRADED SCAFFOLDS

AUTOMATED SYSTEM DEVELOPMENT

The existing algorithm of CASTS only allows the scaffold designer to create scaffolds with uniform porosity. As discussed in Chapter 2, a successful TE scaffold should be functionally graded to facilitate the appropriately seeded cells to attach and proliferate at the desired layer and to perform their functions properly to achieve the regeneration of healthy tissues. This chapter presents the development of the algorithm for designing FGS, for both radial and linear/axial gradients. Radial pore size gradient is useful in the case of regenerating a section of long bones such as a femur, which consists of cortical part in the outer radius and cancellous part in the inner radius. On the other hand, the gradient in linear direction can be used in generating scaffolds for other types of bone, such as the mandible, and tissue interfaces, such as the bone-cartilage interface. Like CASTS, this automated FGS system is built on Pro/Engineer platform.

4.1. Radial FGS

As discussed in Chapter 2, the structural gradients of a long bone, which are the cortical and the cancellous, typically occur radially, where the cortical (the denser part) is located at the outer ring and the cancellous (the more porous part) is at the inner ring. As observed from its cross-section, human bone has a porosity gradient such that the strength and stiffness gradually becomes lower from the outer towards the inner section [23].

Hence, the proposed methodology to achieve the stiffness gradient in the radial direction is by varying the scaffold porosity accordingly. In this method, the pores, represented by spheres, are assembled in the arrangement of concentric rings (refer to Figure 4.1 (a)). When pores with equal sizes are arranged as a ring and their centers are connected together, they will form a regular polygon (N-sided polygon), as seen in Figure 4.1 (b) (6-sided polygon). The regular polygon will have N vertices, where N is the number of pores at that particular ring. Three main parameters for the generation of such structure are the

pore size, radial distance, radian increment and the number of pores at a particular ring. These concentric ring structures will then be stacked to form a concentric cylinder structure.

Figure 4.1. Pores assembled in concentric rings of a cylinder

The representation of the pores using spheres is to simplify the process of establishing the radial FGS parameters and the relations amongst these parameters. Once the spheres' model is established, the spheres are replaced using unit cylinders (shown in Figure 4.2) to create an open-pore scaffold structure, in which the struts act as the cell attachment substrate, while the pores facilitate the nutrient and waste transport, as well as the cell migration. The unit cylinder is an additional structure to be incorporated into the CASTS system and is specially designed to be the repeatable unit cell in the radial FGS model mimicking the repeating structural unit of concentric lamellae making up the compact bone (refer to Figure 4.3). Furthermore, the unit cylinder structure offers practicality in the process of assembling the radial gradient. The shape of the unit cylinder has the flexibility to be assembled into concentric rings, without the need of deforming the structure or adding additional feature to fit them into the concentric ring structure. It also eases the calculation for scaffold volume, surface area, porosity, and hence its stiffness.

Figure 4.2. Unit cylinder as the unit structure for radial FGS

Figure 4.3. Structure of a long bone [157]

4.1.1. Parameters and Relationships of the Sphere Model

The parameters of the ring structure and their relationships are defined and explained as follows:

1. Radial distance of the pores

The tabulation of the radial distance is important to define the position of the pores at a particular ring. The radial distance, RD_n , is measured from the center of the center pore (called pore zero/ring zero) to the center of the pore at current (n^{th}) ring (as shown in Figure 4.4).

Figure 4.4. Schematic diagram describing the radial distance

The radial distance is defined as:

$$RD_n = RD_{n-1} + \frac{P_{n-1}}{2} + \frac{P_n}{2} \quad (\text{Equation 4.1})$$

Where: RD_n = radial distance of pores at n^{th} ring

RD_{n-1} = radial distance of pores at $(n-1)^{\text{th}}$ ring

P_{n-1} = diameter of pores at $(n-1)^{\text{th}}$ ring

P_n = diameter of pores at n^{th} ring

2. Radian increment

Trigonometry rules were applied to calculate the radian increment θ_n (refer to Figure 4.5). In this case, the position of each pore was specified in polar coordinates.

Figure 4.5. Schematic diagram describing the radian increment

The radian increment of pore n , θ_n , is defined as:

$$\sin\left(\frac{\theta_n}{2}\right) = \frac{P_n}{2RD_n}$$

$$\theta_n = 2 \arcsin\left(\frac{P_n}{2RD_n}\right) \quad (\text{Equation 4.2})$$

3. Number of pores at n^{th} ring

When the angle in a circle (2π) is divided by the radian increment (θ_n), the predicted number of pores might have the excess of the radian increment, and this excess will create the presence of void, as shown in Figure 4.6. Therefore, to avoid the generation of voids, the calculated number of pores (N) was rounded up or down, depending on the desired application. When N was rounded down, it would result in increasing pore size towards the outer radius, while rounding N up would result in decreasing pore size.

Figure 4.6. Presence of void in the ring structure

For increasing pore size in the outward radial direction:

$$N_n = \left\lfloor \frac{2\pi}{\theta_n} \right\rfloor \quad (\text{Equation 4.3})$$

Where: N = the number of pores at n^{th} ring

For decreasing pore size in the outward radial direction:

$$N_n = \left\lceil \frac{2\pi}{\theta_n} \right\rceil \quad (\text{Equation 4.4})$$

4. Number of layers at n^{th} ring in the cylinder

The number of stacked layers at n^{th} ring in the cylinder can be calculated by rounding up the value obtained from the dividing the cylinder height with the pore size.

$$L_n = \left\lceil \frac{h}{P_n} \right\rceil \quad (\text{Equation 4.5})$$

Where: L_n = the number of layers in the cylinder at n^{th} ring in the cylinder
 h = the height of the cylinder

With the size of the center pore (P_0), concentric cylinder diameter (D), and concentric cylinder height (H) as the inputs (shown in Figure 4.7), through several iterations using the above relationships the pores can be assembled into a concentric cylinder structure. The isometric and plan views of the assembled pores are presented in Figure 4.8, which shows decreasing pore sizes towards the outer radius.

Figure 4.7. User inputs for the sphere model

Figure 4.8. Isometric and plan views of the sphere model for radial FGS

4.1.2. Parameters and Properties of the Unit Cylinder

Before going into the details on replacing the spheres using the unit cylinders, the parameters of the unit cylinder needs to be defined. Each unit cylinder comprises of two halves of a torus and four vertical cylindrical struts connecting the two halves of the torus. The three parameters of the unit cylinder are the distance between the center of the unit cylinder and the center of the torus tube (R), the radius of the torus tube (r), and the height

of the unit cylinder (H) (refer to Figure 4.9). The radius of the cylindrical struts is the same as the value of r.

Figure 4.9. The defining parameters of the unit cylinder

To calculate its theoretical porosity (void volume fraction), the volume of the unit cylinder must be first established. The unit cylinder consists of two halves of a torus and four vertical cylindrical struts; hence its volume can be defined as:

$$V_{\text{unit cyl}} = 2.V_{\text{ht}} + 4(V_s - V_i) \quad (\text{Equation 4.6})$$

Where: V_{ht} = volume of a half torus = $\pi^2.R.r^2$

V_s = volume of a cylindrical strut = $\pi r^2.H$

V_i = volume of an intersection between the strut and the torus

The volume of intersection between one cylindrical strut and the two halves of the torus (or equal to the volume of intersection between one cylindrical strut and one whole torus), represented by V_i in Equation 4.6, can be approximated using an intersection between two identical cylinders, as shown in Figure 4.10 (a). Such intersection consists of eight identical cylindrical wedges (Equation 4.8), each of which has a radius and a height of r, equal to the radius of the cylindrical strut (refer to Figure 4.10 (b)).

Figure 4.10. (a) Two cylinders intersecting at right angle and the shape of its intersection [158], (b) a cylindrical wedge, one eighth the volume of the intersection

By taking integral over the interval from 0 to r, the volume of the wedge shown in Figure 4.10 (b) can be obtained as follows:

$$V_{\text{wedge}} = \int z(x).y(x).dx \quad (\text{Equation 4.7})$$

$$z(x) = x$$

$$y(x) = \pm\sqrt{(r^2-x^2)}$$

$$\text{Hence: } V_{\text{wedge}} = \frac{2}{3} r^3 \text{ and the intersection volume } V_i = 8V_{\text{wedge}} \quad (\text{Equation 4.8})$$

And by substituting Equation 4.8 into Equation 4.6, the volume of a unit cylinder is defined as:

$$V_{\text{unit cyl}} = 2\pi^2.R.r^2 + 4(\pi r^2.H - \frac{16}{3} r^3) \quad (\text{Equation 4.9})$$

The surface area of the unit cylinder can then be obtained by differentiating the unit cylinder volume with respect to r:

$$A_{\text{unit cyl}} = 4\pi^2.R.r + 4(2\pi r.H - 16r^2) \quad (\text{Equation 4.10})$$

As shown in Figure 4.11 (a), the unit cylinders will be arranged in a concentric manner and the total volume V_t is described by the area within the boundary of the dashed lines shown in Figure 4.11 (b). This area can be approximated by replacing the curved dashed lines in Figure 4.11 (b) with a dashed square shown in Figure 4.11 (c). Hence, the porosity of individual unit cylinder can be calculated as follows:

$$\text{Porosity} = 1 - (V_{\text{unit cyl}}/V_t) \quad (\text{Equation 4.11})$$

Where: V_t = total volume consisting of $V_{\text{unit cyl}}$ and void volume inside the enclosure of individual unit cylinder = $4H(R+r)^2$

Figure 4.11. (a) Cylinder units arranged in concentric manner, (b) a zoomed in image of a cylinder unit, (c) the approximation of void space surrounding a cylinder unit

4.1.3. Process Flow of Automating the Design of Radial FGS

The process flow of the radial FGS design automation using the unit cylinders is summarized in Figure 4.12. The detailed discussion on the radial FGS algorithm will be presented in Section 4.1.4.

Figure 4.12. Schematic diagram of the process flow for automating the radial FGS design

In order to simulate a tissue with stiffness gradient, the scaffold is divided into regions with varying stiffness values, in which each region represents one stiffness value. For example, the schematic diagram of the cortical and cancellous regions of a bone scaffold can be described as shown in Figure 4.13. The letter d denotes the diameter of the cancellous region and t denotes the thickness of the cortical region, according to the target bone data. The required scaffold height, h , is determined according to the size of the bone defect. These dimensions, along with the stiffness values for both cortical and cancellous regions, are the inputs to this FGS system.

Figure 4.13. Schematic representation of cancellous diameter, cortical thickness, and scaffold height

The system then translates the stiffness input values into actual scaffold porosity via the stiffness-porosity relations discussed in Chapter 6. The actual porosity is the measured porosity of the fabricated scaffold, which has slight difference from the intended design porosity. The design porosity is the theoretical porosity calculated with the assumption that the scaffold struts are non porous and the scaffold dimensions are fabricated accurately. The actual and design porosities were linearly related and empirical relations were obtained to predict the actual porosity when the design porosity was known, and vice versa.

Via such empirical relations (briefly discussed in Section 6.3.1), the design scaffold porosity is obtained from the actual porosity. The strut size of the unit cylinder, r , can then be obtained via structural relations discussed in section 4.1.2, while the torus radius R is kept constant.

Once the unit cylinder parameters are obtained, the individual unit cylinders can then be generated, positioned and patterned according to the user input. The result will be a radial FGS with cortical and cancellous stiffness variation mimicking the target bone. Boolean operation can then take place between the generated scaffold file and the shape of bone defect for a proper fit.

4.1.4. Algorithm for Automated Radial FGS Generation

The radial FGS model presented in this section comprises of cancellous and cortical regions. Like the sphere model discussed in section 4.1.1, the radial FGS generation using the unit cylinders starts from the center towards the outer part of the concentric cylinder structure. For a better understanding of the model, a schematic description of the concentric cylinder structure and its parameters, namely unit cylinder height (H), scaffold height (h), unit cylinder size (Dc_n), ring diameter (Dr_n), and number of layers (L) are presented in Figure 4.14.

Figure 4.14. Schematic diagram of various parameters of the concentric cylinder structure

- Cancellous ring 0 (the unit cylinder at the center of the concentric structure)

Through porosity-stiffness relations discussed in Chapter 6 and structural relations between porosity and strut diameter, the stiffness value for the cancellous region is translated into a desired strut diameter ($2r$), while the R value is set to be 1 mm. The unit cylinder size, Dc_0 , for ring 0 is defined as its total diameter, which is:

$$Dc_0 = 2(R_0 + r) \quad (\text{Equation 4.12})$$

And the unit cylinder height, H_0 , is set to be the same as the unit cylinder size:

$$H_0 = 2(R_0 + r) \quad (\text{Equation 4.13})$$

After the three parameters (r , R_0 , and H_0) have been defined, the unit cylinder at ring 0 can then be generated. The parameters for locating and patterning the unit cylinder are initiated as follows:

- a. The radial distance for the ring 0, RD_0 , is 0 mm.
- b. The radian increment $\theta_0 = 0$ radian.
- c. The number of unit cylinders at ring 0, $N_0 = 1$.
- d. The number of layers $L = \text{ceiling}(h/H_0)$.

The number of layers at subsequent rings will be the same as L as the unit cylinder heights at subsequent rings are set to be the same as H_0 . When scaffold height is larger or equal to the user input, h , the addition of the subsequent layer is stopped.

- e. The ring diameter of ring 0, Dr_0 , is the same as the unit cylinder size Dc_0 . The subsequent diameter of ring n is defined as:

$$Dr_n = Dr_{n-1} + 2 Dc_n \quad (\text{Equation 4.14})$$

This is a parameter required as a control once the input cancellous or cortical region size has been achieved. When ring diameter is larger or equal to the required region size, then the addition of the subsequent ring is not needed and stopped.

- Cancellous ring 1 (the first series of unit cylinders arranged in a concentric ring surrounding ring 0)

- a. The assumed unit cylinder size for ring 1, Dc'_1 is assumed to be the same as Dc_0 .
- b. The assumed radial distance is (adopted from Equation 4.1):

$$RD'_1 = RD_0 + Dc'_1/2 + Dc_0$$

- c. The assumed radian increment is (adopted from Equation 4.2):

$$\sin\left(\frac{\theta'_1}{2}\right) = \frac{Dc'_1}{2RD'_1}$$

$$\theta'_1 = 2 \arcsin\left(\frac{Dc'_1}{2RD'_1}\right)$$

- d. From Equation 4.4, the number of unit cylinders is:

$$N_1 = \frac{2\pi}{\theta'_1}$$

Notice that N_1 is always an integer when unit cylinder size Dc'_1 is the same as Dc_0 , imposing that the ring 1 consists of exactly six unit cylinders without creating any voids. Therefore, it is not required to round up the number and Dc_1 is equal to Dc'_1 , while RD_1 is equal to RD'_1 .

- e. The diameter of ring 1 is $Dr_1 = Dr_0 + 2 Dc_1$.

- Cancellous ring 2

- a. The assumed unit cylinder size Dc'_2 is set to be the same as Dc_1 .

- b. The assumed radial distance $RD'_2 = RD_1 + Dc'_2/2 + Dc_1$

- c. The assumed radian increment is:

$$\theta'_2 = 2 \arcsin\left(\frac{Dc'_2}{2RD'_2}\right)$$

- d. The number of unit cylinders is:

$$N_2 = \left\lceil \frac{2\pi}{\theta'_2} \right\rceil$$

At ring 2, an exact integer number cannot be obtained for N_2 when Dc'_2 is the same as Dc_1 , indicating that there will be a void generated. Hence, it is necessary to round the number to eliminate the void and iteration is needed to obtain the values of Dc_2 and RD_2 . In order to obtain lower porosity towards the outer radius like native long bone, N_2 is rounded up.

- e. The actual radian increment $\theta_2 = 2\pi/N_2$

- f. The actual unit cylinder size Dc_2 is:

$$\sin\left(\frac{\theta_2}{2}\right) = \frac{Dc_2}{2RD_2} \quad (a), \text{ and:}$$

$$RD_2 = RD_1 + Dc_2/2 + Dc_1 \quad (b)$$

There are two equations, (a) and (b), and two unknowns, the actual unit cylinder size Dc_2 and the actual radial distance RD_2 . Hence, by substituting (b) into (a) and inputting the value of θ_2 , one can obtain the values of Dc_2 and RD_2 . According to these values, the location and dimension of individual unit cylinders in this ring are specified. Likewise, the process was repeated for the subsequent rings.

g. The diameter of ring 2 is $Dr_2 = Dr_1 + 2 Dc_2$.

- At each cancellous ring, checks were carried out to establish whether the required cancellous diameter (according to the patient's data as input by the user) has been achieved by the concentric cylinder structure. If the required diameter has not been achieved, subsequent cancellous rings were continuously added until the desired value was achieved. When the required cancellous region diameter has been obtained, the addition of cancellous rings was stopped and the generation of cortical region started.

- **Cortical ring**

According to the input cortical stiffness and via empirical and structural relations, the strut diameter ($2r$) for the unit cylinder in the cortical region was obtained. The calculation of the concentric cylinder structure parameters like the radial distance, radian increment, etc. followed and continued from those in the cancellous region. The additional of cortical rings stopped when the input cortical thickness has been achieved.

As a summary, by having the stiffness values of the cortical and cancellous bone as the user input to the automated FGS system and by using the relations derived in Chapter 6 suitable strut diameter for both the cortical and cancellous regions were obtained. Both regions can then be generated, in which the cancellous region was enclosed in a shell of cortical region in a concentric manner. After this radial FGS has been completed (refer to Figure 4.15), it is time for Boolean operation to take place between the generated radial

FGS and the shape of the target bone defect. The resulting scaffold will have radial stiffness gradient with cancellous and cortical regions mimicking the target bone and the overall shape of the bone defect for a proper fit onto the defect site.

Figure 4.15. Plan and isometric views of the radial FGS model using repeatable unit cylinders

4.1.5. Verification of Radial FGS System

To verify the feasibility of the automated system, a radial FGS for a femur segment was created as an example. The process flow of the customized FGS generation is described in Figure 4.16.

Firstly, the data of the femur segment was gathered, namely the femur segment image, the cortical and cancellous region size, and the stiffness values for both regions. The femur segment image was obtained from CT scan and was processed using MIMICS to get an IGES file, which can be imported and manipulated using Pro/Engineer. The femoral shaft diameter and cancellous region diameter are 3.14 cm and 2.30 cm, respectively [159]. The femoral cortical and cancellous stiffness are 17 GPa and 441 MPa, respectively [160]. These stiffness values, especially the cortical stiffness, far exceeded the feasible PCL and PCL/HA scaffold stiffness discussed in Chapter 6. However, this shortcoming can be overcome by immobilizing the patient using cast until the fixation between the scaffold

and the surrounding bone has been stabilized. After that, the cast can be removed and replaced using functional bracing, which allows patient's mobilization to encourage osteogenesis [161-162]. Another option is material range extension to include polymers or composites with higher stiffness, which is discussed in the future work (Section 8.2).

Hence, in this case, the actual values of cortical and cancellous stiffness were not used. Instead, to illustrate the stiffness variation in the radial FGS, two stiffness values that are within the range of PCL stiffness were used, namely, 65 and 20 MPa for the cortical and cancellous regions, respectively. These region dimensions and stiffness values were then input to the system. According to the porosity-stiffness relations for the unit cylinder stated in Table 6.4, these stiffness values give the scaffold porosity variation of 65% and 80% for the cortical and cancellous regions, respectively. The suitable strut diameters were then generated to obtain the radial FGS.

After that, the femur segment was superimposed onto the radial FGS and Boolean operation took place in order to obtain a graded scaffold with the shape of the femur segment. The customized radial FGS were then fabricated using SLS and the fabricated scaffold is shown in Figure 4.17.

Figure 4.16. Process flow of generating customized radial FGS

Figure 4.17. The fabricated radial FGS for femur defect

4.2. Linear FGS

Besides long bone regeneration using radial FGS, other bone categories that do not have longitudinal axes such as the scapulae and mandible can be regenerated using scaffolds with linear gradients. The proposed method for automating the design of linear FGS is shown in Figure 4.18. In this schematic model, regions with differing stiffness were positioned side by side. To facilitate matching of different regions, the unit cell size was kept constant for all regions, while the strut diameter was varied to account for stiffness gradient.

Figure 4.18. Schematic diagram of linear FGS

The process flow of the linear FGS design automation followed what was described in section 4.1.3. Briefly, user inputs to the system included the desired stiffness values and the size of each region. The system then translated the stiffness inputs into appropriate strut diameter through a series of formulas relating the scaffold stiffness, porosity, and structural parameters. After that the unit cells can then be positioned, and patterned to get the FGS according to the user input. Finally, a Boolean operation between the generated FGS file and the shape of bone defect would complete the process of obtaining the customized linear FGS.

4.2.1. Parameters and Relationships

The parameters and properties of individual unit cells in the CASTS system include the unit cell size, strut diameter, unit cell volume and surface area and have been established by Chua *et al.* [21-22] and Naing *et al.* [11]. On the other hand, the parameters that define the linear FGS are depicted in Figure 4.19. They are namely: the region size in x-y-z direction, the number of unit cells, and the spacing in between two neighboring unit cells.

Figure 4.19. Schematic diagram of the parameters of a region in linear FGS

The detailed discussion on the parameters of the linear FGS is as follows:

1. Number of unit cells

The number of unit cells, N , in a stack is differentiated into three according to x-y-z axes. These three numbers are denoted by N_{x_n} , N_{y_n} , and N_{z_n} , where N_x is the number of unit cells along the x axis, while N_y and N_z are along y and z axis, respectively. The subscript n represents the region number, e.g. region 1, 2, and so on.

These three numbers are obtained from the user input for the region size. Rounding adjustment is done to make sure that the scaffold block is slightly larger than required in order to accommodate for the Boolean operation between the FGS block and the defect image. Hence, the number of unit cells is defined as:

$$N = \left\lceil \frac{L'}{H} \right\rceil \quad (\text{Equation 4.15})$$

Where: L' = region size input
 H = unit cell size

2. Region size

The final region size, L , is obtained from multiplying the number of unit cells with the unit cell size ($L = N \times H$). It is also differentiated according to x-y-z axes, L_{x_n} , L_{y_n} , and L_{z_n} , where the subscript n denotes the region number.

3. Unit cell spacing

The unit cell spacing, SP , plays a role in defining the position of a unit cell in a region block. It is the distance between the centers of two neighboring unit cells and its value is the same as the unit cell size H .

4.2.2. Algorithm

The automation process flow is as follows:

- Region 1

When the stiffness input is captured, the system provides the user with a list of available CASTS configurations that can match the stiffness input. After a desired configuration is selected, the stiffness input for the region 1 is translated into a corresponding strut diameter ($2r$) according to the porosity-stiffness relations discussed in Chapter 6 and structural relations between the porosity and strut diameter of the selected configuration. The first unit cell for region 1 is then generated and positioned. According to user input on

the region size, the number of unit cells in all three directions, N_{x1} , N_{y1} , and N_{z1} , are calculated and the unit cells are generated, forming a block for region 1.

- Region 2

Like the first unit cell in region 1, the first unit cell in region 2 is generated with appropriate strut diameter derived according to the stiffness input. Its position along x axis is defined by region 1 length in the x direction, L_{x1} , while its y and z positions follow those of the first unit of region 1, as shown in Figure 4.20. Next, to form a block for region 2 the number of unit cells in all three directions, N_{x2} , N_{y2} , and N_{z2} , are calculated and the unit cells are generated and patterned according to the calculated numbers. The generation of the subsequent regions follows these steps until a complete and desired FGS block is obtained, as shown in Figure 4.21. Boolean operation between the generated FGS block and the bone defect image will follow afterwards.

Figure 4.20. Defining the position of the first unit of region 2

Figure 4.21. Plan and isometric views of the linear FGS model

4.2.3. Verification of Linear FGS System

The feasibility of the linear FGS system was verified using a case study of creating a FGS for human mandible. From Misch *et al.* [77], there exists a stiffness gradient along the cancellous bone of human mandible, which can be divided into three regions as shown in Figure 4.22. Region 1 includes the incisors and canine with compressive stiffness of 67.48 MPa, region 2 includes the premolars with stiffness of 47.30 MPa, and region 3 includes the molars with stiffness of 35.55 MPa. Each region was measured to be approximately 20 mm long (along x direction), 35 mm wide (along y direction) and 40 mm tall (along z direction). These dimensions were crucial to size the individual scaffold blocks for each region.

Figure 4.22. Top view of a human mandible

After all the required stiffness and dimensions have been input, a FGS block was generated using configuration 10 (octagonal and square prism) of CASTS system (refer to Table 5.2 in Chapter 5 for the full list of configuration numbers and their corresponding polyhedral types) and PCL/HA material to mimic the gradients of the mandible. Firstly, in order to calculate the PCL/HA scaffold porosity for each region, the porosity-stiffness relations for configuration 10 (refer to Table 6.5) was used. The scaffold porosity values obtained from the relation were 45%, 50% and 53%, respectively for regions 1, 2 and 3. Structural relation between the porosity and strut diameter for configuration 10 was also used to obtain the required strut diameter in order to generate the scaffold blocks for each region. Individual scaffold blocks were then stacked to form a FGS block. The defect image, in this case the mandible segment, was then superimposed onto the FGS block. The mandible was assumed to be symmetrical, hence, only the regeneration of one half of the mandible was performed. The Boolean operation was then carried out to obtain the intersection of the FGS block and the mandible. The result was a scaffold with stiffness gradient with an overall shape of a human mandible. These steps are summarized in Figure 4.23. The fabricated mandible scaffold is shown in Figure 4.24.

Figure 4.23. Process flow of generating customized linear FGS

Figure 4.24. The fabricated linear FGS for mandible defect

4.3. Summary

In this chapter, the parameters, process, and algorithm for automating the design of radial and linear FGS have been developed. The feasibility of the automated FGS system has also been verified using case studies of femur and mandible FGS. This radial and linear FGS system will serve as an attractive aid for tissue scaffold designer in designing scaffolds with gradients mimicking the target organ. Although the automated system is already fully functional, there is still room for future improvement. For example, the methodology can be extended to include biodegradable polymers or composites that can fulfill the high stiffness requirement for cortical bone to complete the database of the system. This will be highlighted in the future work.

Chapter 5 – EXPERIMENTAL PROCEDURES

This chapter discusses the procedures of experiments carried out throughout the research, including the determination of SLS sintering parameters, both for PCL and PCL/HA powder, SLS-fabricated parts' characterization (in terms of structural integrity, accuracy, and surface roughness), and mechanical characterization of CASTS designed scaffolds. Cell culture and maintenance protocols are explained, along with the methods on cell seeding onto the scaffolds and analysis in terms of cell attachment, proliferation, and mineralization.

5.1. Determining Suitable Sintering Parameters

5.1.1. SLS Parameters for Sintering PCL Powder

Before proceeding to build the scaffolds, one needs to determine the suitable SLS parameters to build scaffolds with good quality, including rigidity and accuracy. This section presents the experiments carried out to obtain the suitable parameters, including laser power, scan speed and part bed temperature.

Tan *et al.* [136] has specified the optimum laser scan speed and part bed temperature for sintering PCL powder as shown in Table 5.1. The laser power was given in a range, hence there was a need to find a suitable laser power value.

Table 5.1. Optimum SLS parameters for sintering PCL powder [136]

SLS Parameters	Values
Laser power	2-4 Watts
Scan speed	3810 mm/s (150 in/s)
Part bed temperature	40 °C

These criteria were taken into consideration during the selection of the suitable laser power:

1. Micro features structural integrity

Structural integrity is important, as the scaffold should maintain its structure and shape during fabrication, cleaning and handling. This criterion was analyzed using visual observation and also scanning electron microscope (SEM) to ensure that the scaffold micro features are still intact after cleaning and handling.

2. Accuracy

As the scaffold will have structures in micrometer range, it is important to investigate the SLS resolution and the effect of varying SLS parameters on part accuracy.

To obtain the suitable laser power, the rest of the SLS parameters, namely part bed temperature and scan speed, were kept constant while the laser power was varied. This is based on the work by Tan *et al* [136]. At part bed temperature of 40 °C and scan speed of 3810 mm/s, sintering PCL powder using laser power less than 2 W resulted in very porous parts, which were difficult to handle, while at above 4 W the sintered parts became warped, making it more difficult to sinter tall parts with many layers, as the warped layers shifted and deformed the part's shape [136]. Therefore, in this experiment the laser power was varied at 2 W, 2.5 W, 3 W and 3.5 W, while keeping the part bed temperature at 40 °C and scan speed at 3810 mm/s.

Two types of part were built using varying laser power to address the structural integrity and accuracy:

1. A rectangular block with micro ridges (refer to Figure 5.1), three samples each, to be viewed under SEM to analyze the effect of laser power on part accuracy. Prior to the SEM analysis, the samples were gold-coated using JEOL JFC-1600 AutoFine Coater with 20 mA current for 150s. After coating, the samples were viewed under JEOL JSM-5600LV scanning electron microscope. The ridges dimensions (750 µm, 500 µm, and 250 µm) were checked for intactness and then measured using SMile View software by JEOL (SMV). SMV works by pasting a micron bar calibrated to a size of image to measure the size of a particular feature.

2. A tall rectangular block, five samples each, to assess the warpage issue, which will affect scaffold shape and accuracy, and feasibility of building tall parts. The blocks were $12.7 \times 12.7 \times 25.4 \text{ mm}^3$.

Figure 5.1. A rectangular block with micro patterns (t_1 = ridge thickness for region 1, t_2 for region 2, and t_3 for region 3). $t_1 = 750 \text{ }\mu\text{m}$, $t_2 = 500 \text{ }\mu\text{m}$ and $t_3 = 250 \text{ }\mu\text{m}$.

5.1.2. SLS Parameters for Sintering PCL/HA Powder

SLS parameters meant for sintering PCL alone may not be suitable for sintering PCL/HA powder as HA, being a ceramic material, requires very high temperature to sinter ($>1000^\circ\text{C}$) [163-164] and hence could not be sintered during the fabrication process. Increasing laser power to increase the HA powder temperature to the point of sintering was not an option as the high temperature will degrade the PCL material (degradation point around 350°C) [165]. Therefore, the PCL particles were used as a binder to bind the unsintered HA particles.

With the same laser power and part bed temperature as obtained in section 5.1.1, the laser scan speed was varied in this experiment to allow the PCL powder to bind the HA particles stronger. Three rectangular blocks with micro ridges (refer to Figure 5.1) were

fabricated using laser scan speed of 1270, 1905, 2159, 2540, and 3810 mm/s (50, 75, 85, 100, and 150 in/s). Part characteristics analyzed included structural integrity and accuracy.

5.2. Characterization of SLS-Sintered Scaffolds

The parts fabricated via SLS were characterized in terms of part accuracy and surface roughness. The part accuracy was examined to determine the smallest feature the SLS can build. On the other hand, the surface roughness of the SLS sintered PCL powder was also quantified. The SLS parameters used in these experiments such as part bed temperature, laser power and laser scan speed were determined from the previous experiments (refer to section 5.1).

5.2.1. Scaffold Resolution

The measurement of the smallest feature the SLS can sinter was carried out as explained in section 5.1.1 on the fabrication of the rectangular blocks with micro ridges and the measurement of the fabricated micro ridges.

5.2.2. Surface Roughness

To examine the surface roughness of the sintered PCL powder, cylindrical disks of 15 mm diameter and 2 mm thickness were fabricated using SLS, in three replicates. The surface roughness of each disk was then quantified using Talyscan 250 Dual Gauge System. Talyscan 250 possesses contact and non-contact analysis modes. As the contact mode involves a small probe tracing the surface of the sample, it will cause damage to the PCL sample surface. Hence, the non-contact (laser gauge) mode was chosen. This mode required reflective samples. Therefore, the samples were gold-coated using JEOL JFC-1600 AutoFine Coater with 20 mA current for 150 s. Each sample was placed below the laser source and the scan speed was set to be 4500 $\mu\text{m/s}$. The area to be measured was set to be 2 x 2 mm.

5.3. Effect of Porosity and Structure on Compressive Stiffness and Strength

To design FGS with a stiffness gradient mimicking bone, it is necessary to have a complete database of the scaffold properties that can be tailored to meet the required stiffness value. As it has been known that scaffold structure and porosity directly affect stiffness, the objective of this experiment is to formulate relationships between scaffold porosity and its compressive stiffness, unique to each CASTS polyhedral structure/configuration, including the additional unit cylinder structure for the radial FGS generation. In order to obtain such relationships, scaffolds with different polyhedral structures were designed to have various porosity values to be fabricated using SLS, both using PCL and PCL/HA powder, and then compressive tested. Then, calculated compressive stiffness values were plotted against their corresponding scaffold porosity to observe the trend and relationship between the two variables for each of the scaffold configuration. Empirical relationships were then obtained to model the trends, which were further implemented into the FGS automation program discussed in Chapter 4.

The scaffolds were designed using CASTS system (configuration number and polyhedral types are listed in Table 5.2) with the overall scaffold dimension set to be 25.4 x 25.4 x 12.7 mm. The configuration details of CASTS system are described in Chua *et al.* [21-22]. By adjusting the strut diameter between 0.6-1.0 mm, three porosity values were set for each configuration, namely 40%, 60%, and 80%.

Table 5.2. Configuration number and polyhedral type of CASTS-designed scaffolds

C	Polyhedral Type	C	Polyhedral Type
1.	Octahedron and tetrahedron	9.	Hexagonal prism
2.	Cuboctahedron and octahedron	10.	Octagonal prism and square prism
3.	Triangular prism	11.	Truncated cuboctahedron and hexagonal prism
4.	Square prism		
5.	Square pyramid	12.	Hexagonal prism and triangular prism
6.	Rhombic dodecahedron	13.	Square prism and triangular prism
7.	Truncated octahedron	14.	Unit cylinder
8.	Truncated cube and octahedron		

Note: C stands for configuration number.

To obtain the compressive stiffness and yield strength, all samples were tested using Instron 5569 Universal Tester machine. Testing parameters were set according to ASTM D 1621-04a, Standard Test Method for Compressive Properties of Rigid Cellular Plastics [166].

5.4. Cell Culture and Maintenance Protocols

In vitro experiments were carried out to observe the response of living cells to fabricated scaffolds. Before going into the detail of these experiments, the cell culture protocols and cell maintenance will be discussed. The cell type used in this research was human fetal osteoblast (hFOB 1.19), purchased from American Type Culture Collection (ATCC).

5.4.1. Cell Growth Cycle

A typical cell growth cycle can be divided into three phases [167], as shown in Figure 5.2, which are:

1. The lag phase

This is the phase where there is a lag before growth begins when the cells are first seeded onto a substrate. During this time the cells have to attach themselves to the substrate and spread out, thus the expansion rate is comparatively slower than the log phase.

2. The log phase

During this phase, the cells are growing and the population is doubling. It is the most suitable time for the cells to be used in experiments. The length of this phase depends on the initial cell seeding density.

3. The plateau phase

When the cells have covered the surface of the substrate (become confluent), the growth rate slows down and may eventually stop. It is time for the tissue culturist to subculture the cells onto new substrate to bring the growth phase back to the lag phase in order to avoid overcrowded substrate. Overcrowded substrate is not a healthy

environment for the cells, causing a decrease in the cell number as some of the cells may die.

Figure 5.2. A typical cell growth cycle: (a) the lag phase, (b) the log phase and (c) the plateau phase

Upon receiving a vial of frozen cells from the supplier, the vial was immediately stored inside a liquid nitrogen tank. When it was time to start culturing the cell, the frozen cells were resuscitated by rapidly thawing them and subsequently the cells were grown as a monolayer culture in tissue culture flasks. The culture was checked once every three days and the medium was replaced with the fresh one. When the cells reached about 80% confluency, they were subcultured into some new flasks. Quality control was done to ensure that the culture was free from contamination such as fungi, bacteria, yeasts and mycoplasma. After the quality control has been done, the cells were ready to be used for seeding onto scaffolds. Some of the cells were frozen to allow for banking purposes. In case of contamination, the contaminated cells should be disposed of and a new batch of frozen cells was resuscitated. The process flow of cell culture and maintenance described is illustrated in Figure 5.3.

Figure 5.3. Process flow in cell culture and maintenance

5.4.2. Equipments and Reagents

With regards to health and safety, the cell culture works were carried out in a Gelman Class II Biosafety cabinet with vertical laminar flow, to establish protection for both the cell culturist and the work. The cultures were stored inside a JISICO J-2000 incubator, which was set at 36.5 °C temperature and 4.0% CO₂ level. During the cell culture works, a pair of rubber gloves and a lab coat were worn at all times.

Basic reagents required for the culturing the cells are classified as follows:

1. Cell culture medium

The main ingredients of the cell culture medium are inorganic salts, amino acids, vitamins and buffers. The inorganic salts are important for cells metabolism, while the amino acids and vitamins provide the essential nutrients required for cell growth and division. The buffers provide a counterattack measure to avoid rapid change in medium pH due to the large amount of acidic waste products from the cells metabolism. As a pH indicator, phenol red is usually added as a visual aid to detect the change of pH in the medium. The pH level of cell culture medium is normally

between 7.2 -7.4 and the color is deep red. The indicator spans the pH range between 6.8 (yellowish)-8.4 (purple) [167-168].

2. Medium Supplements

Foetal Bovine Serum (FBS) is usually added to the medium due to its high content of growth factors needed for cell growth. Besides FBS, antibiotics are usually included in the cell culture medium to inhibit microorganism contamination [167-168].

3. Phosphate Buffered Saline (PBS)

PBS is an isotonic buffer solution with pH 7.4, containing sodium chloride (84%), sodium hydrogen phosphate anhydrous (12%), potassium phosphate (2%) and potassium chloride (2%). It is non-toxic to the cells and helps maintain constant pH in the culture. It is usually used for washing and cleaning the cells from waste and cell debris [169].

The complete growth medium for osteoblasts (hFOB 1.19) was Dulbecco's Modified Eagle Medium with F-12 nutrient mixture (DMEM/F-12) (Invitrogen, catalog no. 11320033) supplemented with 10% heat-inactivated FBS (Invitrogen, catalog no. 16140063) and 0.3 mg/ml G418 Geneticin (Invitrogen, catalog no. 10131027) as the antibiotic [170].

5.4.3. Subculturing

When the culture was approximately 80% confluent, the cells were ready to be subcultured. The old medium was removed from the flasks and the cells were washed using PBS. Ten milliliter of fresh complete medium was added into each flask after the removal of PBS. The cells were detached from the flasks surface using cell scrapers and resuspended to ensure uniform cell distribution. The subcultivation ratio was 1:4 [170]. A balanced amount of medium was then added into the new flasks.

5.4.4. *Cell Resuscitation and Freezing*

In cell resuscitation, frozen vial of cells was rapidly thawed in a 37°C water bath with gentle agitation. Vial contents were transferred into centrifuge tube with fresh complete medium and centrifuged at 125 x g for 5 minutes. The supernatant was carefully removed, fresh complete medium was added and the cells were resuspended. After resuspension, the entire contents of the tube were transferred to a prepared flask and incubated [170].

To freeze cells for banking purposes, cells were detached from the flask surface and transferred into a centrifuge tube. The tube was centrifuged, and the supernatant was removed and replaced using cryoprotectant medium containing complete growth medium with additional 20% FBS and 8% (v/v) dimethyl sulfoxide (DMSO) [170]. The tube contents were then resuspended and transferred into cryovials. The cryovials were stored in –80°C freezer overnight before being transferred into liquid nitrogen.

5.4.5. *Contamination Detection*

Contamination by bacteria, fungi and yeast could be easily detected by a sudden drop of pH or the turbidity of the culture medium. The sudden drop of pH was caused by the rapid growth of the contaminants and would change the color of the medium to yellow. If the culture medium became cloudy or the color suddenly changed from red to yellow overnight, it was likely that the culture was contaminated. These kinds of contamination could also be checked under the microscope.

Bacteria appear like dots smaller than the cells, floating on the medium. Fungi appear filamentous and are easy to observe under low microscope magnification, while yeasts often clump together and are smaller than the cells [167-168].

Mycoplasmas are small microorganisms that lack the rigid cell wall. Mycoplasma infection might affect cellular growth rate, morphology and metabolism. Mycoplasma contamination was not easily observed under the microscope. Thus, it was necessary to

confirm such contamination by staining the cells [167-168]. The staining process was done using Mycoplasma Stain Kit (Sigma, product no. MYC-1) according to the procedure proposed by Sigma [171]. The observation was carried out under a fluorescence microscope (Olympus BX51). Under fluorescence microscope, healthy cells would be stained only on the nucleus, while the contaminated cells would have their cytoplasm stained as well.

5.4.6. Cell Counting

Cell counting was performed using the haemocytometer (Marienfeld, Germany), a modified glass slide with counting grids engraved on it. The grids consisted of nine squares, 1 x 1 mm each with 0.1 mm depth (refer to Figure 5.4). The haemocytometer was used together with a glass coverslip, which was put on top of the haemocytometer [172].

Figure 5.4. Haemocytometer grids/counting chamber [172]

Before counting the cells, the haemocytometer and the cover slide were sterilized using 70% ethanol, rinsed using de-ionized water and dried using tissue paper. After the cells were detached from the flask surface and resuspended, the suspension was transferred and mixed with Trypan Blue Solution (Sigma, catalog no. T8154) to dilute the initial solution by four times. The final mixture was pipetted onto the haemocytometer under the coverslip. The viable cells did not absorb the dye, hence they would appear clear and unstained, while the dead cells were stained blue. The cells within the A, B, C, D and E squares (refer to Figure 5.4) were counted. From cell counting, one could obtain cell density and also the percentage of cell viability as follows:

$$1. \text{ Cell density} = \frac{\Sigma N}{5} \times d \times 10^4 \text{ cells/ml, where:} \quad (\text{Equation 5.1})$$

ΣN = the number of viable cells within squares A-E

d = dilution factor, in this case $d = 4$

$$2. \text{ Cell viability (\%)} = (\text{the number of viable cells/total number of cells}) \times 100\%, \text{ where}$$

the number of viable and total cells were counted within squares A-E.

(Equation 5.2)

5.5. Cell Seeding and Observation Protocols

This section presents the protocol of cytotoxicity test and cell seeding onto the scaffold. Cells and scaffold observation protocols, including cell proliferation assay, SEM observation, and mineralization assay are also discussed.

5.5.1. Cytotoxicity Test

In vitro cytotoxicity test was performed to assess the possible toxic effect of the scaffold leachables to the cells, which could be induced by the scaffold material, fabrication or sterilization process. A negative result suggests that there are no presence toxic leachables or the scaffold has only minimum amount of toxic leachables insufficient to cause adverse reactions to the cells. In contrast, a positive result implies that the harmful leachables from the scaffold cause loss of cell viability.

Complete culture medium was used as a negative control, while an extract from latex rubber gloves was used as the positive control. The procedure of the cytotoxicity test was according to the extract dilution process [173-174], as follows:

1. The scaffolds (both PCL and PCL/HA) and latex rubber were immersed in complete medium at 4 g/ml using separate centrifuge tubes and incubated at 37°C for 72 hours.
2. Cells were seeded on 24-well plates at a concentration of 5×10^3 cells/well and incubated at 37°C for 24 hours.

3. The cell medium was replaced using the extracts from the scaffolds (both PCL and PCL/HA) and the positive and negative controls, which were separately dispensed onto the cell-seeded wells ($n = 5$). The plates were subsequently incubated at 37°C for 72 hours.
4. Cell proliferation assay was performed to evaluate the cell viability and the readings were normalized to the values obtained for the negative control.

5.5.2. Cell Seeding onto Scaffolds

The cell seeding experiments were carried out according to the process flow depicted in Figure 5.5.

Figure 5.5. Process flow of cell seeding onto a scaffold

Before the cells were seeded onto the scaffolds, the scaffolds were sterilized by immersing them in 70% ethanol for 3 hours. The scaffolds were then rinsed using PBS with agitation to remove traces of ethanol. After rinsing, the scaffolds were placed on 24-well plates and immersed in complete medium overnight to condition the scaffolds for the cell seeding.

The procedure of seeding cells onto the scaffolds is as follows:

1. The cells were detached from the flasks surface.
2. The cells from several flasks were transferred into one centrifuge tube and resuspended to ensure uniform cell distribution.
3. Cell counting was performed to determine the cell density of the cell suspension.
4. Based on the cell density, the seeding density was decided and the amount of cell suspension to be seeded was calculated.

5. The old medium in which the scaffolds were immersed overnight was removed and the cells were pipetted onto the scaffolds carefully.
6. The 24-well plates were then put inside an incubator and the medium was replaced every three days.

On the days of observation, the observations performed include cell proliferation assay by MTT staining and examination under SEM. Both observation procedures will be discussed in the next sections.

5.5.3. *Cell Proliferation Assay*

To examine the proliferation of cells seeded on the scaffolds, a colorimetric assay was used. Tetrazolium dye, MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), was used to stain the viable cells. This method relies on the mitochondrial dehydrogenases in living cells to reduce the yellow color MTT to an insoluble, dark blue formazan salt. The colorimetric data correlates well with the number of viable cells [172].

The kit used was Vybrant MTT Cell Proliferation Assay Kit (Invitrogen, catalog no. V13154). The MTT solution was prepared by dissolving MTT powder in sterile PBS solution with final concentration of 5 mg/ml. The solution was then sterilized by filtering. To dissolve the formazan salt, lysing buffer solution was used. This buffer was prepared by dissolving sodium dodecyl sulfate (SDS) powder in a solution of 1:1 deionized water and dimethyl formamide (DMF). The final concentration of the lysing buffer was 0.2 g/ml.

The protocol of MTT staining followed Vybrant MTT Cell Proliferation Assay Kit experiment protocol [175]. The color absorbance measurement was done using Microplate Spectrophotometry (Benchmark Plus) at a wavelength of 570 nm. As the conversion of MTT to formazan was cell-type specific, it was recommended that cell culturists prepare their own color absorbance/cell number calibration curve.

5.5.4. Cell Observation under SEM

Besides examining cell proliferation by MTT staining, one might wish to analyze the cells seeded on the scaffolds under SEM. Below is the procedure:

1. The medium was removed from the cell-seeded scaffolds and the cells were fixed by immersing the scaffolds in glutaraldehyde (GA) solution (2.5% in PBS) overnight.
2. The GA solution was completely removed and the scaffolds were rinsed using PBS.
3. The PBS solution was removed and a series of ethanol dehydration was performed by immersing the scaffolds in 70%, 80%, 90% ethanol for 15 min each, followed by immersing the scaffolds in 100% ethanol 2x15 minutes.
4. The ethanol was removed and the scaffolds were freeze-dried.
5. The scaffolds were then gold-coated and observed under SEM.

5.5.5. ECM Mineralization Assay

In order to compare the mineralization of the osteoblasts seeded on different scaffolds, Alizarin Red S assay was used to stain the mineral secreted by the cells. The reagents required include [176]:

- 20 mM Tris Buffered Saline (TBS) with 0.15 M NaCl, pH 7.4 adjusted with HCl
- 75% (v/v) ethanol, ice-cold
- 40 mM Alizarin Red S in distilled water (DW), pH 4.2 adjusted with 10% (v/v) ammonium hydroxide (NH_3 or NH_4OH)
- 10% (w/v) Cetylpyridinium Chloride (CPC) in 10 mM sodium phosphate (Na_2PO_4)

The staining procedures are as follows [177-178]:

1. The culture (including the substrate/scaffolds with cells) was first washed twice with 20 mM TBS.
2. The culture was then fixed in ice-cold ethanol for 30 minutes, air-dried, and moved to new plates.
3. The culture was stained using 500 μl Alizarin red S for 1 hour and extensively rinsed with water to removed unbound stain.

4. The bound stain was then diluted using 1 ml 10% CPC for 15 minutes.
5. The diluted stain was pipetted to 96-well plates for the color absorbance to be measured using Microplate Spectrophotometry (Benchmark Plus) at a wavelength of 570 nm.

Chapter 6 – RESULTS AND DISCUSSION: SCAFFOLD FABRICATION AND CHARACTERIZATION

This chapter discusses the results of functionally graded scaffold fabrication and characterization, including the suitable sintering parameters for PCL and PCL/HA powders, the resolution and surface roughness of SLS sintered scaffolds, and the porosity-compressive strength and stiffness relationship of the CASTS designed scaffolds. The results validating the feasibility of the automated FGS design system are presented as well.

6.1. Determining Suitable Sintering Parameters

6.1.1. SLS Parameters for Sintering PCL Powder

Based on the laser power range presented by Tan *et al.* [136], the results on the parts sintered using various laser power are as follows:

1. Cylindrical disks

A fabricated cylindrical disk is shown in Figure 6.1. The SEM micrographs of the sintered disk surface are shown in Figure 6.2, where Figure 6.2 (a) displays the surface of a disk sintered using 2 W laser power, Figure 6.2 (b) 2.5 W, Figure 6.2 (c) 3 W and Figure 6.2 (d) 3.5 W. It can be observed that the bonding and the formation of necks (indicated by the white arrows) among the powder particles sintered using 2 and 2.5 W laser power are not that obvious. The necks formation becomes much more prominent in the powder sintered using 3 W laser power (refer to Figure 6.2 (c)), leading to a denser and stronger part. In Figure 6.2 (d) the powder particles sintered using 3.5 W laser power have coalesced to form a polymer melt.

Figure 6.1. A sintered cylindrical disk

Figure 6.2. SEM micrographs of sintered PCL with varying laser power: (a) 2 W, (b) 2.5 W, (c) 3 W and (d) 3.5 W (white arrows indicate the formation of necks among powder particles)

2. Rectangular blocks

The tall rectangular blocks built using varying laser power between 2 W and 3.5 W are shown in Figure 6.3. It was observed that warpage occurs at the bottom of the part built with 3.5 W laser power due to the non-uniform cooling of the sintered layer. This

was because the temperature gradient between the sintered layer and the surrounding loose powder was too large and the higher the laser power, the larger the temperature gradient was. During sintering, the warped layers were often swept by the SLS roller, causing shifting of layers and therefore compromising the part shape and accuracy.

Figure 6.3. Parts built using 2 W, 2.5 W, 3 W and 3.5 W laser power, respectively. Warpage (circled in white) was noted at the bottom of part built using 3.5 W laser power.

3. Rectangular block with micro ridges

To measure the accuracy of the parts sintered using different laser power, rectangular parts with micro ridges (refer to Figure 6.4) were fabricated. The part possessed three regions of ridges with 3 different dimensions of namely 750 μm , 500 μm and 250 μm . After fabrication, the samples were viewed under SEM and the ridges were measured using SMile View Software.

Figure 6.5 pictures the SEM micrograph of 250 μm ridges sintered using different laser power. It can be seen than the ridges sintered using 2 and 2.5 W laser power had some damages due to handling and cleaning, while the ridges sintered using 3 and 3.5 W laser power were intact with good structural integrity. It implies that laser powers of 2 and 2.5 W are not sufficient to keep fine structures stiff enough to prevent damages due to handling and cleaning.

Figure 6.4. A rectangular block with micro patterns (t_1 = ridge thickness for region 1, t_2 for region 2, and t_3 for region 3). $t_1 = 750 \mu\text{m}$, $t_2 = 500 \mu\text{m}$ and $t_3 = 250 \mu\text{m}$.

Figure 6.5. SEM micrographs of $250 \mu\text{m}$ ridges sintered using varying laser power: (a) 2 W, (b) 2.5 W, (c) 3 W and (d) 3.5 W

Figure 6.6 shows the ridges' measurement using SMile View software. The 250 μm ridges sintered using all laser power resulted in averagely 360 μm wide ridges, which indicates that the smallest feature that can be sintered using the SLS is approximately 360 μm . The 500 and 750 μm ridges were sintered well with 87-95 % accuracy, where 2 W laser power produced the most accurate dimension and 3.5 W the least accurate.

Figure 6.6. The measurement of the ridges width using SMile View software

A summary of the effect of varying laser power on the sintered part characteristics is presented in Table 6.1. In general, increasing laser power linearly increases sintered part density and inter-particle bonding while decreasing porosity and accuracy. Sintering using 2 and 2.5 W laser power resulted in parts with low structural integrity that were damaged during handling and cleaning, while 3 and 3.5 W laser power produced parts with good structural integrity. Warpage occurred during the sintering of parts with 3.5 W, resulting in shifted layers and compromised part shape and accuracy. Therefore, 3 W laser power was selected as it could provide parts with good structural integrity while maintaining high level of accuracy. The selected SLS parameters for sintering PCL powder are presented in Table 6.2.

Table 6.1. Summary of the effect of laser power to sintered part characteristics

Laser Power Tested (W)	Structural Integrity	Accuracy (%)	Warpage	Chosen	Remarks
2	Low	95	No	No	Weak inter particle powder bond causes fine structures to be destroyed during cleaning and handling
2.5	Low	94	No	No	Weak inter particle powder bond causes fine structures to be destroyed during cleaning and handling
3	Good	92	No	Yes	Good structure integrity with high accuracy
3.5	Good	87	Yes	No	Warpage causes sintered layers to shift, leading to less accurate part and even failure

Note: The smallest feature the SLS can sinter is approximately 360 μm .

Table 6.2. Optimum SLS parameters for sintering PCL powder

SLS Parameters	Values
Laser power	3 Watts
Scan speed	3810 mm/s (150 in/s)
Part bed temperature	40 °C

6.1.2. SLS Parameters for Sintering PCL/HA Powder

However, sintering PCL/HA 10 wt% using the parameters listed in Table 6.2 resulted in parts with poor mechanical integrity. In order to have more robust parts without degrading the PCL material, the laser scan speed was lowered (1270, 1905, 2159, 2540 and 3810 mm/s or 50, 75, 85, 100 and 150 in/s, respectively) while other parameters were kept constant according to Table 6.2.

It was found that the test specimens that were built at laser scan speed of 1270 mm/s and 1905 mm/s showed visible signs of partial-sintering, a common phenomenon occurring in laser sintering process, where the laser energy penetrates beyond the designed scanning

path [179]. In Figure 6.7, two neighboring ridges sintered using 1905 mm/s scan speed were merged because of the partial sintering effect, which was due to the scan speed being too low. Thus, the surrounding powder that was supposed to be loose and unsintered was being melted or partially sintered.

Figure 6.7. Part sintered using 1905 mm/s scan speed, showing the partial-sintering effect

On the other hand, test specimens that were fabricated at 2540 mm/s and 3810 mm/s resulted in weak structures which disintegrated upon cleaning and handling, indicating that the laser scan speeds were too high. Test specimens that were fabricated at 2159 mm/s did not exhibit the presence of over-sintering while retaining its mechanical integrity during handling and cleaning, as shown in Figure 6.8. Therefore the laser scan speed of 2159 mm/s (85 in/s) was selected. The preferred SLS parameters for sintering PCL-HA specimens are summarized in Table 6.3.

Figure 6.8. Part sintered using 2159 mm/s scan speed, showing clearly defined ridges

Table 6.3. Optimum SLS parameters for sintering PCL/HA powder

SLS Parameters	Values
Laser power	3 Watts
Scan speed	2159 mm/s (85 in/s)
Part bed temperature	40 °C

6.2. Characterization of SLS-Sintered Scaffolds

6.2.1. Scaffold Resolution

It was observed that ridges with dimension of 300 μm and below could not be sintered accurately. Instead, such ridges were sintered with final ridge width of 350 μm . This means that the dimension of the minimum feature that can be sintered using SinterStation 2500 is around 350 μm (refer to Figure 6.5). This result corresponds with the diameter of the SLS laser beam, which is around 400 μm for SinterStation 2500 [14].

6.2.2. Surface Roughness

The average surface roughness (peak-to-valley amplitude) R_a value of sintered PCL disks, which was measured using the laser gauge of Talyscan 250, was 33.7 μm , which was due

to the large particle size of the PCL powder ($\sim 100\ \mu\text{m}$). The three-dimensional visual description of the surface microtopography is shown in Figure 6.9. As seen from Figure 6.9 the peaks/valleys of the roughness appeared to be randomly distributed.

Figure 6.9. Three-dimensional visual description of sintered PCL surface

As reported in some literatures [69, 92], it was stated that such surfaces with microtopography could support osteoblast attachment and mineralization. An increase in rat calvarial osteoblast attachment has been reported to be a function of surface roughness, while less mature cells could undergo osteogenic differentiation when seeded on rough surface [92]. Schneider *et al.* found that osteoblasts experienced enhanced mineralization when grown on roughened implant surfaces with microtopography relative to smooth tissue culture plastic [69]. However, no surface roughness value was quantified in these works. Hence, to assess the suitability of the achieved surface roughness for tissue engineering application, *in vitro* experiment was carried out to examine cell adhesion on the sintered PCL (refer to Section 7.5).

6.3. Effect of Porosity and Structure on Compressive Stiffness and Strength

Besides facilitating tissue ingrowth and matching the defect shape, it is important that the scaffold for bone applications possesses similar mechanical properties with the target bone to avoid stress concentration from occurring [8-9, 13, 78]. Mismatch of mechanical properties between the scaffold and the bone could cause stress shielding when the scaffold is stiffer than the bone, or scaffold failure when the scaffold has inferior mechanical properties to the bone [28, 78]. Thus, mimicking the stiffness variation across the bone, FGS for bone tissue regeneration should have a similar gradient in terms of the mechanical properties, specifically compressive stiffness [19].

While there is no certain standard available yet on how close one should mimic the bone stiffness gradient, it is advisable to closely mimic the gradient to avoid either scenario mentioned above. The degree of similarity of the FGS stiffness gradient to the native bone depends on several factors including the currently available biomaterial, scaffold design, fabrication and testing technology. Starting from obtaining the patient's data, there is a certain limit of accuracy the mechanical test can provide. In designing the FGS to mimic the native bone, the degree of similarity also depends on the FGS model accuracy. Finally, during the fabrication process, there is also a limit to the accuracy of the fabrication technology available. Therefore, within the available technology and resources, one should try to mimic the stiffness gradient as closely as possible to the native bone.

FGS with stiffness variation can be achieved by varying its porosity with respect to the spatial position as scaffold porosity directly contributes to its compressive stiffness [61]. Thus, in order to establish relationship between porosity and compressive stiffness, compression tests were performed on the thirteen CASTS configurations with varying porosity. Two types of powder, PCL and PCL/HA, were used as the scaffold material.

6.3.1. General Observations and Stress-Strain Behaviour of CASTS Scaffolds

Ten out of 14 CASTS configurations (refer to Table 5.2) were used to construct the scaffolds, as configuration 7 (truncated octahedron) was unable to form scaffold with 40% porosity value, while both configurations 8 (truncated cube and octahedron) and 11 (truncated cuboctahedron and hexagonal prism) were unable to form scaffold with 40% and 60% porosity values. This was caused by the overlapping of adjacent struts when increasing the strut diameter to lower the porosity value. Figure 6.10 shows that even at 80% porosity, configuration 8's struts were almost overlapping. Lowering the porosity to 60% and 40% increased the strut diameter, hence causing the struts to overlap. Thus, Pro/Engineer was not able to generate the scaffold files.

Figure 6.10. Almost overlapping struts seen in configuration 8 (truncated cube and octahedron) at 80% porosity

The actual scaffold porosity (V_p), defined as the percentage of void volume in a scaffold, was calculated according to the following equation [180]:

$$V_p = 1 - \frac{\rho^*}{\rho_s} \quad (\text{Equation 6.1})$$

Where:

ρ^* = density of the scaffold = (scaffold mass) g / (2.54 x 2.54 x 1.27) cm³

ρ_s = density of the strut material

It was observed that the actual scaffold porosity deviated from the intended/designed porosity because of these following reasons:

1. The designed struts were assumed to be solid, where in fact the SLS-fabricated scaffold struts were porous.
2. The intended strut dimension was assumed to be accurately fabricated, where in fact the actual fabricated strut dimension had slight deviation from the designed dimension due to partial sintering effect. Partial sintering is a phenomenon in which the laser energy dissipates from the sintering path to the surrounding loose powder, partially sintering the loose powder outside the sintering path.
3. The scaffolds with low porosity had trapped loose powder because of the difficulty of removing the supporting powder.

Depending on the scaffold structure, the porosity deviation varied for different configurations. Hence, for each configuration the actual scaffold porosity was plotted against the intended design porosity and trend lines with empirical equations were added. A common measure of correlation, R^2 (squared of the Pearson correlation coefficient) values were calculated for each empirical equation to measure how closely the equation represented the data points. The results showed that the actual scaffold porosity was linearly related to the design/intended porosity with strong correlation ($R^2 > 0.9$), which meant that the empirical relations can represent the nature of the data points well.

The scaffolds were then compressed until 60% strain and typical stress-strain curves for CASTS scaffolds were plotted, as shown in Figure 6.11. Generally, all scaffold configurations behave like typical cellular solids under compression with three clearly defined trends in the stress-strain curve, marking the linear-elastic region (region I), a plateau of constant stress (region II), and steeply rising region (region III). At the linear-elastic region, the individual struts deform elastically. When the strain increases, the struts begin to bend plastically, constructing a plateau of constant stress. The plateau region ends when the adjacent struts begin to collapse, come into contact to one another and

compact the sample. This increases the load needed to compress the now compact sample, giving the steep rise at the end of the stress-strain curve [181].

Figure 6.11. Typical stress-strain curves for CASTS scaffolds

As the strut diameter increases, the porosity decreases, and the densification region comes earlier as the struts will come into contact with each other faster, making the plateau region shorter. In scaffolds with low porosity the plateau region is so short that it is less obvious. Decreasing the porosity increases the stiffness of the samples, as marked by the increase of the elastic region steepness. These trends can be observed in the stress-strain plots of all tested CASTS configurations, both PCL and PCL/HA scaffolds, which are attached in Appendix page 181-190.

Compressive stiffness of the scaffold (E^*) was defined as the scaffold ability to resist compressive deformation within the linear range and was computed as the ratio of stress to corresponding strain below the proportional limit, while compressive strength (σ_c) was defined as the stress at yield point if it occurs below 10% strain, or in the absence of such

yield point, the stress at 10% strain [166]. The factors affecting the stiffness include the scaffold material, porosity, strut aspect ratio and strut shape/configuration. The stresses computed were based on the force applied divided by the overall cross-sectional area of the sample, including the void pore area, and the samples were evaluated as a whole. The rule of mixtures (see section 6.3.2) was used to predict the relationships between the scaffold porosity and compressive stiffness, while curve plots of experimental data (porosity-stiffness) were used to verify the relationship prediction.

6.3.2. *The Rule of Mixtures*

A widely accepted micromechanical model, the rule of mixtures, was used to predict the stiffness of the sintered scaffolds. It is generally used for predicting the stiffness of composite materials, in which composite stiffness lies in between the stiffness values of the individual components of the composite. With more than one phase/material present, the sintered PCL and PCL/HA scaffolds are considered as composite materials. PCL scaffold has the sintered PCL and voids, making a two-phase composite, while PCL/HA scaffold is a three-phase composite with the PCL, HA and voids as its components.

As the starting point, the general form of the rule of mixtures assumes that the composite is reinforced with unidirectional continuous fibers and is loaded along the fiber direction (refer to Equation 6.2). Other types of reinforcement, e.g. short fibers and particulates, as well as the arrangement of the reinforcements can be accounted for using correction factors introduced into the general stiffness model. The correction factors, which were experimentally determined, took into account the different arrangements of the scaffold struts (different CASTS configuration leads to different strut arrangement/orientation), as well as the effect contributed by the sintering process (imperfections/micro pores) to the scaffolds stiffness. How such correction factors were added into the general model is discussed in Section 6.3.3 for PCL scaffolds and Section 6.3.4 for PCL/HA scaffolds.

The porosity-stiffness relationship prediction started with general model for unidirectional and continuous fiber-reinforced matrix as follows [182]:

$$E_c = V_f \cdot E_f + V_m \cdot E_m \quad (\text{Equation 6.2})$$

Where: E = stiffness

V = volume fraction

Subscripts c, f, and m denote the composite, fiber and matrix respectively.

When there is no porosity accounted:

$$v_c = v_f + v_m \quad (\text{Equation 6.3})$$

$$v_f = m \cdot W_f / \rho_f$$

$$v_m = m \cdot W_m / \rho_m \quad (\text{Equations 6.4})$$

Where: v = volume

m = total composite mass

W = weight fraction

ρ = density

Substituting Equations 6.4 into Equation 6.3:

$$v_c = (m \cdot W_f / \rho_f) + (m \cdot W_m / \rho_m) \quad (\text{Equation 6.5})$$

$$\text{Since } V_f = v_f / v_c \quad (\text{Equation 6.6})$$

Substituting Equations 6.4 and 6.5 into Equation 6.6:

$$V_f = \rho_m \cdot W_f / (\rho_f \cdot W_m + \rho_m \cdot W_f) \quad (\text{Equation 6.7})$$

$$\text{Similarly, since } V_m = v_m / v_c \quad (\text{Equation 6.8})$$

Substituting Equations 6.4 and 6.5 into Equation 6.8:

$$V_m = \rho_f \cdot W_m / (\rho_f \cdot W_m + \rho_m \cdot W_f) \quad (\text{Equation 6.9})$$

Where: $V_f + V_m = 1$ (Equation 6.10)

Now, taking scaffold porosity into account, the rule of mixtures was modified as presented by Madsen *et al.* [183]:

The total volume of the scaffold is:

$$V_c = V_f + V_m + V_p \quad (\text{Equation 6.11})$$

Where: Subscript p denotes the porosity.

$$\begin{aligned} V_f &= m \cdot W_f / \rho_f \\ V_m &= m \cdot W_m / \rho_m \\ V_p &= V_p \cdot V_c \end{aligned} \quad (\text{Equations 6.12})$$

Substituting Equations 6.12 into Equation 6.11:

$$\begin{aligned} V_c &= (m \cdot W_f / \rho_f) + (m \cdot W_m / \rho_m) + V_p \cdot V_c \\ V_c \cdot (1 - V_p) &= (m \cdot W_f / \rho_f) + (m \cdot W_m / \rho_m) \\ V_c &= [(m \cdot W_f / \rho_f) + (m \cdot W_m / \rho_m)] / (1 - V_p) \end{aligned} \quad (\text{Equation 6.13})$$

$$\begin{aligned} V_f &= V_f / V_c \\ V_f &= \rho_m \cdot W_f \cdot (1 - V_p) / (\rho_f \cdot W_m + \rho_m \cdot W_f) \\ V_m &= V_m / V_c \\ V_m &= \rho_f \cdot W_m \cdot (1 - V_p) / (\rho_f \cdot W_m + \rho_m \cdot W_f) \end{aligned} \quad (\text{Equations 6.14})$$

Where: $V_f + V_m + V_p = 1$ (Equation 6.15)

Substituting Equations 6.14 into Equation 6.2:

$$E_c = V_f \cdot E_f + V_m \cdot E_m$$

$$E_c = [\rho_m \cdot W_f / (\rho_f \cdot W_m + \rho_m \cdot W_f)] \cdot (1 - V_p) \cdot E_f + [\rho_f \cdot W_m / (\rho_f \cdot W_m + \rho_m \cdot W_f)] \cdot (1 - V_p) \cdot E_m$$

(Equation 6.16)

But: $\rho_m \cdot W_f / (\rho_f \cdot W_m + \rho_m \cdot W_f) = V_f$, and

$$\rho_f \cdot W_m / (\rho_f \cdot W_m + \rho_m \cdot W_f) = V_m$$

when porosity was not accounted for (refer to Equations 6.7 and 6.9).

Hence:

$$E_c = (V_f \cdot E_f + V_m \cdot E_m) \cdot (1 - V_p)$$

(Equation 6.17)

Where: $V_f + V_m = 1$

Equation 6.17 was used to predict the compressive stiffness of PCL and PCL/HA CASTS scaffolds, as presented in sections 6.3.3 and 6.3.4.

6.3.3. PCL CASTS Scaffolds

The scaffolds fabricated using SLS were found to be consistent with the CAD drawing and able to maintain their structural integrity and withstand the stresses of the handling and cleaning process (see Figure 6.12). The polyhedral-based CASTS scaffolds in the ideal case should have fully interconnected pores. However, pore occlusion was observed for scaffolds with 40% porosity due to partial sintering effect. The pore size was reduced and loose powder became trapped. Nevertheless, the pores of scaffolds with 60% and 80% porosity were observed to be interconnected and most of the loose powder was removed from the samples.

Figure 6.12. CAD file and its corresponding fabricated PCL scaffold

Scaffold porosity was calculated using Equation 6.1 with PCL density ($\rho_{\text{PCL}} = 1.153 \text{ g/cm}^3$) as the ρ_s (density of strut material). The scaffold porosity, compressive stiffness and strength range from 40-86%, 2.74-95.18 MPa and 0.17-7.32 MPa respectively.

Compressive stiffness-porosity relationships were predicted using the rule of mixtures. For PCL scaffolds, which consist of only two constituents, PCL struts and porosity (void fraction), the model in Equation 6.17 can be reduced into:

$$E_c = (V_f \cdot E_f) \cdot (1 - V_p) \quad (\text{Equation 6.18})$$

Where: $V_f = 1$

Accounting for the strut orientation variation, a correction factor, C_1 [184], was added into Equation 6.18:

$$\begin{aligned} E_c &= C_1 \cdot E_f \cdot (1 - V_p) \\ E_c &= -C_1 \cdot E_f \cdot V_p + C_1 \cdot E_f \\ E^* &= -C_1 \cdot E_{\text{PCL}} \cdot V_p + C_1 \cdot E_{\text{PCL}} \end{aligned} \quad (\text{Equation 6.19})$$

Curve fittings were done to data points relating porosity and compressive stiffness for each CASTS configuration. It was found that stiffness is inversely related to porosity in

linear fashion (shown in Figure 6.13) with strong correlation ($R^2 > 0.90$), validating Equation 6.19. Through curve fittings the value of C_1 was then obtained for each CASTS configuration.

Figure 6.13. A plot of scaffold porosity against compressive stiffness for PCL scaffold

The value of C_1 is unique to each scaffold configuration. At the same porosity, different configuration/structure has different stiffness values, as depicted in Figure 6.14. In Figure 6.14, the porosity and stiffness values of configurations 1 and 12 were plotted and compared. At 40% porosity the stiffness of configuration 12 reaches 54 MPa, while configuration 1 only reaches 31 MPa. One possible reason of such 20 MPa stiffness difference is because configuration 12 consists of hexagonal and triangular prisms, which have vertical struts that can act as columns to bear the load with direction parallel to its struts, whereas configuration 1 is constructed of octahedrons and tetrahedrons, which have slanted (non-vertical) struts like truss structures that fail by strut bending. It was found that the top four stiffest configurations possess struts oriented as columns, namely configuration 14 (unit cylinder), 12 (hexagonal and triangular prism), 10 (octagonal and

square prism) and 9 (hexagonal prism). The porosity-stiffness relations of the fabricated scaffold configurations are presented in Table 6.4. These relations were then incorporated into the algorithm discussed in Chapter 4.

Figure 6.14. Comparison of trend lines between two PCL scaffold configurations

Table 6.4. Porosity-stiffness relations of the fabricated PCL scaffolds

Configuration	Polyhedral Type	Relation
1	Octahedron and tetrahedron	$E^*_1 = -67.5 V_p + 58.2$
2	Cuboctahedron and octahedron	$E^*_2 = -78.9 V_p + 67.2$
3	Triangular prism	$E^*_3 = -49.2 V_p + 43.1$
4	Square prism	$E^*_4 = -68.1 V_p + 58.7$
5	Square pyramid	$E^*_5 = -49.5 V_p + 42.2$
6	Rhombic dodecahedron	$E^*_6 = -77.8 V_p + 60.7$
9	Hexagonal prism	$E^*_9 = -86.5 V_p + 75.3$
10	Octagonal prism and square prism	$E^*_{10} = -122.2 V_p + 102.0$
12	Hexagonal prism and triangular prism	$E^*_{12} = -136.6 V_p + 108.9$
13	Square prism and triangular prism	$E^*_{13} = -58.9 V_p + 53.7$
14	Unit cylinder	$E^*_{14} = -273.0 V_p + 242.2$

When the compressive stiffness data was plotted against compressive strength, it was observed that the relationship between the compressive stiffness and strength of CASTS-designed scaffolds is rather linear (as shown in Figure 6.15). This relationship applies regardless of the scaffold structure and porosity, which is in line with the findings by Woesz *et al.* [185]. In the work by Woesz *et al.*, repeatable cellular units having three different strut configurations were fabricated using SLS with polyamide as the material and then compressive tested. Their findings were summarized into two points. First, it was found that the variation of stiffness and strength can be achieved by varying the scaffold strut configuration. Second, there was linear correlation between stiffness and strength for all samples regardless their porosity and strut configuration due to the dependence of both stiffness and strength on porosity [185].

Figure 6.15. A plot of PCL scaffold compressive stiffness against compressive strength

Combining the porosity-stiffness curves of all configurations, the feasible porosity and stiffness ranges were obtained for CASTS-designed PCL scaffolds sintered using SLS (as shown by the shaded region in Figure 6.16).

Figure 6.16. The feasible porosity and compressive stiffness range (shaded region) for CASTS-designed PCL scaffolds sintered using SLS

6.3.4. PCL/HA CASTS Scaffolds

The fabricated PCL/HA scaffolds, as shown in Figure 6.16, were observed to be consistent with the CAD drawing. Although a few samples of 80% porosity were damaged during the handling and cleaning process, most of the scaffolds were able to maintain their structural integrity and hence were sent for compression test. Configuration 1 scaffolds were excluded from the compression test due to excessive layer shifting during fabrication, resulting in distorted scaffold shapes.

Figure 6.17. CAD file and its corresponding fabricated PCL/HA scaffold

The PCL/HA scaffolds were made of 90 wt% PCL and 10 wt% HA. The density of PCL/HA, $\rho_{s \text{ PCL/HA}}$, was calculated as follows:

$$\rho_{s \text{ PCL/HA}} = w_t / v_t \quad (\text{Equation 6.20})$$

where:

w_t = total weight of the composite powder = $w_{\text{PCL}} + w_{\text{HA}}$

v_t = total volume of the composite powder = $v_{\text{PCL}} + v_{\text{HA}}$

hence:
$$\rho_{s \text{ PCL/HA}} = (w_{\text{PCL}} + w_{\text{HA}}) / (v_{\text{PCL}} + v_{\text{HA}})$$

substituting $v_{\text{PCL}} = w_{\text{PCL}} / \rho_{s \text{ PCL}}$ and $v_{\text{HA}} = w_{\text{HA}} / \rho_{s \text{ HA}}$:

$$\rho_{s \text{ PCL/HA}} = (w_{\text{PCL}} + w_{\text{HA}}) / (w_{\text{PCL}} / \rho_{s \text{ PCL}} + w_{\text{HA}} / \rho_{s \text{ HA}})$$

substituting $w_{\text{PCL}} = 0.9 w_t$ and $w_{\text{HA}} = 0.1 w_t$:

$$\rho_{s \text{ PCL/HA}} = (\rho_{s \text{ PCL}} \cdot \rho_{s \text{ HA}}) / (0.9 \rho_{s \text{ HA}} + 0.1 \rho_{s \text{ PCL}})$$

substituting $\rho_{s \text{ PCL}} = 1.153 \text{ g/cm}^3$ and $\rho_{s \text{ HA}} = 3.050 \text{ g/cm}^3$:

$$\rho_{s \text{ PCL/HA}} = 1.229 \text{ g/cm}^3$$

The calculated density of PCL/HA, $\rho_{s \text{ PCL/HA}}$, was then used in the scaffold porosity calculation according to Equation 6.1.

The scaffold porosity, compressive stiffness and yield strength range from 31-83%, 0.85-117.32 MPa and 0.01-9.91 MPa, respectively. The relationship between scaffold porosity and compressive stiffness was then predicted using the rule of mixtures.

In the case of PCL/HA scaffolds, there are three constituents involved, namely, PCL that acts as the binder, HA that acts as particle reinforcement, and the porosity. Equation 6.17 was adjusted to involve strut orientation correction factor, C_1 , and HA particle distribution correction factor, C_2 .

$$E_{\text{scaffold}} = C_1 \cdot (C_2 \cdot V_{\text{HA}} \cdot E_{\text{HA}} + V_{\text{PCL}} \cdot E_{\text{PCL}}) \cdot (1 - V_p) \quad (\text{Equation 6.21})$$

In an attempt to validate the model in Equation 6.21, experimentally obtained stiffness values were plotted against the PCL/HA volume fraction ($1 - V_p$) and curve fittings were performed to obtain the empirical relationships. It was found that stiffness increases with PCL/HA volume fraction ($1 - V_p$) according to power law (refer to Figure 6.18). This could be due to the stress concentration resulting from the porosity and the unsintered HA particles. In samples with high porosity (and correspondingly low ($1 - V_p$)), the strut size was small, hence, the pores and unsintered HA particles became stress concentrator, which disrupted the bond between adjacent PCL particles, significantly decreasing the compressive stiffness. Hence, porosity correction exponent, n , was included to account for the stress concentration [184]. The general equation is as follows:

$$E_{\text{scaffold}} = C_1 \cdot (C_2 \cdot V_{\text{HA}} \cdot E_{\text{HA}} + V_{\text{PCL}} \cdot E_{\text{PCL}}) \cdot (1 - V_p)^n \quad (\text{Equation 6.22})$$

Figure 6.18. A plot of PCL/HA volume fraction (1- V_p) against compressive stiffness for PCL/HA scaffolds

As the strut orientations follow those of the PCL scaffolds, the values of C_1 for all PCL/HA scaffolds follow C_1 values for PCL scaffolds. The values of C_2 should be the same for all configurations as C_2 represents the HA particle orientation and distribution in the PCL binder. Calculations were performed to check the uniformity of C_2 values and it was found that C_2 values are in close range, between 0.1-0.3. The high correlation ($R^2 > 0.95$) between Equation 6.22 and the data point distribution confirmed that Equation 6.22 is a good estimate to predict PCL/HA CASTS scaffold stiffness provided the scaffold porosity is known. The porosity-stiffness relationships of the fabricated PCL/HA scaffolds are listed in Table 6.5.

Table 6.5. Porosity-stiffness relationships of the fabricated PCL/HA scaffolds

Configuration	Polyhedral Type	Relation
2	Cuboctahedron and octahedron	$E_2^* = 298.9(1-V_p)^{3.3}$
3	Triangular prism	$E_3^* = 364.0(1-V_p)^{3.5}$
4	Square prism	$E_4^* = 157.7(1-V_p)^{3.0}$
5	Square pyramid	$E_5^* = 512.6(1-V_p)^{4.7}$
6	Rhombic dodecahedron	$E_6^* = 317.4(1-V_p)^{3.9}$
9	Hexagonal prism	$E_9^* = 477.4(1-V_p)^{4.1}$
10	Octagonal prism and square prism	$E_{10}^* = 740.5(1-V_p)^{4.0}$
12	Hexagonal prism and triangular prism	$E_{12}^* = 435.4(1-V_p)^{3.5}$
13	Square prism and triangular prism	$E_{13}^* = 200.2(1-V_p)^{2.8}$
14	Unit cylinder	$E_{14}^* = 1028.8(1-V_p)^{3.2}$

As shown in the case for PCL scaffolds, the compressive stiffness and strength of PCL/HA scaffolds correlate linearly as depicted in Figure 6.19. Furthermore, it was also found that the top four stiffest PCL/HA configurations possess struts oriented as columns, namely configuration 14 (unit cylinder), 12 (hexagonal and triangular prism), 10 (octagonal and square prism), and 9 (hexagonal prism), corresponding to those of PCL scaffolds. The feasible porosity and compressive stiffness range for CASTS-designed PCL/HA scaffolds is shown as the shaded region in Figure 6.20.

Figure 6.19. A plot of PCL/HA scaffold compressive stiffness against compressive strength

Figure 6.20. The feasible porosity and compressive stiffness range (shaded region) for CASTS-designed PCL/HA scaffolds sintered using SLS

6.4. Summary

In this chapter, the suitable sintering parameters for PCL and PCL/HA materials have been investigated, including SLS part bed temperature, laser power and laser scan speed, which are summarized in Tables 6.2 and 6.3. These parameters were used for building all of the scaffolds in this project. It was found that using the optimum sintering parameters, features smaller than 350 μm in dimension cannot be built using SLS. This finding is consistent with the SLS laser resolution, which is around 400 μm .

The effect of scaffold structure and porosity on its compressive stiffness and strength was discussed for both PCL and PCL/HA materials. In the case of PCL scaffolds, compressive stiffness decreases linearly with increasing scaffold porosity. On the other hand, stiffness increases with PCL/HA volume fraction (1-porosity) according to power law, which could be due to the stress concentration resulting from the porosity and the unsintered HA particles. These porosity-stiffness relationships for both PCL and PCL/HA materials could be predicted using modified rule of mixtures.

Last but not least, it has been shown that by varying the scaffold structure and porosity, different values of stiffness could be obtained. This was the basis for establishing the automated FGS system discussed in Chapter 4. These porosity-stiffness relationships have been integrated into the system, in which their role is to calculate the required scaffold porosity in order to achieve the stiffness input specified for each FGS region. The detailed discussions on the FGS automation process has been elaborated in Sections 4.1.3 – 4.1.5 for radial FGS (femur case study) and Sections 4.2.2 – 4.2.3 for linear FGS (mandible case study).

Chapter 7 – RESULTS AND DISCUSSION:

***IN VITRO* STUDIES ON SLS SINTERED SCAFFOLDS**

This chapter presents the results of the *in vitro* experiments carried out to observe the response of living cells to fabricated scaffolds, such as cytotoxicity effect, cell proliferation and ECM mineralization. Human fetal osteoblast (hFOB 1.19) cells were used to assess the scaffold suitability for bone tissue engineering applications.

7.1. Human Fetal Osteoblast (hFOB 1.19) Cells

Osteoblasts are cells that constantly remodel bone tissue. Besides synthesizing osteoid, which is unmineralized bone matrix, osteoblasts are also responsible for the mineralization of bone matrix [186]. Osteoblasts attach to the bone ECM *in vivo*, which is rich in various proteins such as type I collagen, fibronectin and vitronectin. The ECM and the nature of adhesion of osteoblasts to various substrates play an important role in modulating bone cell functions [187]. Mineralization could be achieved in 3D *in vitro* culture by preserving the environment so as to mimic the original tissue with regard to cell-cell and cell-ECM relationship [188]. It has been shown that the human fetal osteoblast (hFOB 1.19) cells are able to create mineralized nodules *in vitro*, hence hFOB 1.19 is the appropriate cell line for assessing the scaffold suitability for bone tissue engineering [189].

7.2. Contamination Detection

Before conducting any *in vitro* experiment, the cells were examined to ensure that there was no contamination that can affect the experimental results. The culture medium was visually checked that its color did not change drastically from red into yellow overnight and contaminants such as bacteria, fungi and yeasts were not found when the culture was observed under microscope.

As mycoplasma contamination could not be easily detected visually, a mycoplasma test was carried out and the fluorescence images of the cultures were taken and examined. The fluorescence images of positive and negative controls are shown in Figure 7.1 (a) and (b) respectively. As seen from the positive control image, not only the nuclei, but also the cytoplasm around the nuclei were stained blue. In contrast, the negative control had only the nuclei stained blue. Looking at Figure 7.1 (c), where no cell cytoplasm was stained blue, it was concluded that the osteoblast culture was free from mycoplasma infection.

Figure 7.1. Mycoplasma test fluorescence images: (a) positive control, (b) negative control, and (c) osteoblast culture (negative)

7.3. Cell Density Calibration Curve

After the cells were detached from the flasks surface and counted, hFOB 1.19 cells were seeded on 96-well plates with different cell densities to prepare for the calibration curve (shown in Table 7.1). After seeding, the cells were left overnight to allow them to attach onto the well surface and the following day, MTT proliferation assay was performed and the readings of optical density (OD) were plotted against cell density.

Table 7.1. Seeding density and MTT optical density for calibration curve of hFOB 1.19 (n = 5)

Cells/ml	1×10^4	4×10^4	7×10^4	1×10^5	4×10^5	7×10^5	1×10^6	1.25×10^6
MTT OD	0.042	0.126	0.096	0.153	0.326	0.775	1.022	1.064

The plotted calibration curve is shown in Figure 7.2. The cell density corresponds linearly with the color absorbance with strong correlation ($R^2 > 0.9$). The line equation for the plot is expressed on the figure. This equation was used as a reference to calculate the approximate cell density for the cell culture experiments.

Figure 7.2. MTT calibration curve for osteoblasts

7.4. Cytotoxicity Test

To assess whether the scaffolds have any possible toxic leachables derived from the material, fabrication or sterilization process, *in vitro* cytotoxicity test was performed on both the PCL and PCL/HA scaffolds. Complete medium was used as a negative (non-toxic) control, while an extract from rubber gloves was used as the positive (toxic) control. A negative result suggests that the scaffold does not release toxic leachables or has only minimum amount of leachables, such that it is insufficient to cause adverse reactions to the cells. In contrast, a positive result confirms that the harmful leachables from the scaffold cause loss of cell viability.

The test result was presented in terms of relative cell viability, shown in Figure 7.3. The average cell number for the negative control was taken as 100% cell viability, while the cell viabilities for the scaffold extracts and the positive control were calculated relative to those of the negative control. It is shown that the cell viability for the negative control was significantly different ($p < 0.05$) from the positive control. The loss of cell viability was due to the toxic leachables from the rubber glove. On the other hand, there were no obvious differences between the cell viabilities of both PCL and PCL/HA extracts and the negative control, implying that both PCL and PCL/HA scaffolds were non-toxic and could support cell proliferation.

Figure 7.3. The relative cell viability of cells cultured in PCL and PCL/HA scaffold extracts (negative control cell viability was taken as 100%) (n = 4)

7.5. Effect of Porosity Variation on Cell Proliferation and ECM Mineralization

To study the effect of scaffold porosity on cell proliferation and matrix mineralization, three types of configuration 6 (rhombic dodecahedron) scaffolds with different porosity were tested, namely 40% (6-40), 60% (6-60) and 80% (6-80) (shown in Figure 7.4). The scaffolds were 15 mm in diameter and 2 mm thick and fabricated using PCL. PCL/HA scaffolds were not tested as the HA particles interfered with the matrix mineralization readings using Alizarin Red S. Even without cells, the PCL/HA scaffold was stained red due to the calcium content of the HA.

Figure 7.4. Plan and isometric views, as well as the fabricated samples of (a) 6-40, (b) 6-60 and (c) 6-80 scaffolds

After sterilized using ethanol, the scaffolds were placed in 24 well-plates, ready for cell seeding. The cells were seeded with seeding density of 6×10^5 cells/ml. As a control, cells were seeded on polystyrene wells (PS) without the presence of scaffolds. The cells were maintained for 28 days with 4 observation days (day 1, 6, 14 and 28). The medium was changed every 2-3 days. The analyses performed on the seeded cells included cell proliferation assay, matrix mineralization assay and SEM observation.

The experimental setup on 24 well-plates is shown in Figure 7.5. On a plate called the ‘MTT plate’, the scaffolds located in columns 1-4 were for the MTT cell proliferation assay, while those in column 6 were for SEM observation. Another plate was called the ‘Alizarin Red S (ARS) plate’, in which the scaffolds in columns 1-4 were for the ARS mineralization assay and the wells in columns 5-6 were left empty. Proliferation assay

was performed on all four observation days, while ECM mineralization assay was carried out on day 14 and 28. Hence, there were a total of four MTT plates and two ARS plates in this observation.

Figure 7.5. *In vitro* experimental setup on 24 well-plates

The picture of the cell-seeded scaffolds stained using MTT and ARS on days 14 and 28 is shown in Figure 7.6. The MTT stained the cells dark blue/purple, while the ARS stained the calcified ECM red. As observed from the figure, on day 14 both MTT and ARS color intensity did not show any significant difference in terms of color intensity. However, on day 28, scaffold 6-40 seemed to have significantly darker stain compared to scaffolds 6-60 and 6-80 for both MTT and ARS assays. These visual observations were confirmed by the spectrophotometer readings plotted in Figures 7.7 (MTT reading) and 7.8 (a) (ARS reading).

Figure 7.6. The picture of the cell-seeded scaffolds, stained using MTT and ARS on day 14 and 28

Figure 7.7 shows the cell proliferation over the course of 28 days. It was observed that the seeding efficiency of all scaffolds was low ($<50\%$) (refer to day 1 readings). The higher the porosity, the fewer the cells attached to the scaffold during seeding and high amount of cells dropped to the bottom of the well. This could be because there were more pores on scaffolds with higher porosity, allowing the cells to slip through the pores to the bottom of the well.

Figure 7.7. Cell proliferation on scaffolds with varying porosity (40%, 60% and 80%) over 28 days with cells grown on PS well as the control (n = 4)

Before day 28, there were no significant differences on the cell number of all scaffolds. On day 28, the cell growth has stopped for scaffolds 6-80. However, the cells on scaffolds 6-40 and 6-60 were still proliferating with higher number of cells on scaffolds 6-40. This could be due to the higher surface area that the scaffold 6-40 has and it implies that the cell number on a scaffold is proportional to the scaffold surface area [190]. It is shown in Table 7.2 that although scaffold 6-80 has the highest surface area-to-volume ratio, its surface area is the smallest among the three scaffold types.

Table 7.2. Surface area, volume and surface area-to-volume ratio for scaffolds 6-40, 6-60 and 6-80

Scaffold Type	Surface Area (mm ²)	Volume (mm ³)	Surface Area / Volume Ratio
6-40	882	205	4.30
6-60	804	128	6.28
6-80	723	75	9.64

The ECM mineralization result was plotted in terms of ARS color absorbance/optical density (OD) measured using a spectrophotometer. Figure 7.8 (a) shows that for all scaffolds, the calcium deposited by the cells is proportional to the number of the cells on the scaffolds [191]. Although the control PS had significantly ($p < 0.05$) more cells than all the scaffolds (refer to Figure 7.7), on day 28 when the ECM has matured and mineralized, it had significantly less calcium deposits than scaffold 6-40. Figure 7.8 (b) plots the amount of the calcium deposit (in terms of ARS OD) normalized with respect to the number of cells (in terms of MTT OD). It was shown that all scaffolds have significantly higher calcium deposit-to-cell number ratio than those of PS control with scaffolds 6-80 being the highest. This implies that 3D scaffold structure is necessary for osteoblast cell functions, such as ECM deposition and mineralization.

It was noticed that although the cell number on scaffolds 6-80 has decreased on day 28, the calcium deposits was increased. It could be explained that cell proliferation of osteoblast is functionally related to the synthesis and mineralization of the ECM, where the proliferation stops when the osteoblasts were trapped and embedded in the mineralizing ECM [192].

Figure 7.8. Calcium deposition on all scaffolds and control in terms of (a) ARS OD and (b) ARS OD normalized to the number of cells (represented by MTT OD)

Sample SEM images of the PCL scaffold with and without cells are shown in Figure 7.9. PCL scaffold without cells is shown in Figure 7.9 (a) as a control. On day 6, as pointed by the arrows, it was shown that the cells were able to attach, spread and stretch to bridge the

micro pores between powder particles (Figure (7.9 (b)). On day 14, it can be seen in Figure 7.9 (c) that the cells not only formed interconnected network on the scaffold surface (indicated by the solid arrows), but they were also able to infiltrate the micro pores in between the powder particles (indicated by the dashed arrows). Finally, on day 28 multiple cell layers were formed on the surfaces of the scaffold (Figure 7.9 (d)). As time progressed, the cells became easier to find and more cell layers were formed, which is consistent with the MTT proliferation results discussed previously. It shows that SLS-sintered scaffolds could provide a favorable environment for the cells to attach, proliferate and deposit mineralized matrix for a prolonged period.

Figure 7.9. SEM images of: (a) PCL scaffold without cells, (b) cells bridging the micro pore (indicated by the arrows) on day 6, (c) cell layer on the scaffold (solid arrows) and cells in the micro pores (dashed arrows) on day 14 (d) cell layer covering the scaffold surface on day 28

7.6. Summary

In this chapter, *in vitro* studies of osteoblasts (hFOB 1.19) on PCL and PCL/HA scaffolds have been presented. It was confirmed that the cells cultured were free from contamination before doing any experiments. Cell calibration curve was plotted to relate the MTT optical density (OD) to the number of cells per ml of medium. It was found that the MTT OD correlates linearly with the cell density with high R^2 value. This calibration curve was used to approximate the cell density on the experiments carried out.

Before the cells were seeded onto the scaffolds, cytotoxicity test was performed on both PCL and PCL/HA scaffolds to assess the possible harmful effect of the scaffold leachables. The result showed that both PCL and PCL/HA scaffolds were non-toxic to cells and could support cell proliferation.

The effects of scaffold porosity on cell proliferation and ECM mineralization were investigated using PCL scaffolds. The result showed that scaffold porosity affected the cell proliferation and function. The cell number on the scaffolds corresponded with the surface area of the scaffold. Although scaffolds with 80% porosity had the highest surface area-to-volume ratio, its surface area was the smallest among the three scaffold types tested, hence they contained the least number of cells. Another finding was that the calcium content of the ECM correlated to the number of cells present on the scaffold. Last but not least, it was shown that three-dimensional scaffold structure is a necessity for cell function, such as ECM deposition and mineralization, reflected by the significantly higher calcium deposit-to-cell number ratio of all scaffolds compared to those of two-dimensional polystyrene well.

Chapter 8 – CONCLUSIONS AND FUTURE WORK

This chapter summarizes and concludes the works carried out throughout this research. Possible future work and further improvements to this project are also presented. Lastly, the publications as the result of this work are listed.

8.1. Conclusions

This research focuses on the development of automated system for designing functionally graded scaffolds (FGS) intended for bone applications. The system runs on Pro/Engineer and the validation was carried out on the Selective Laser Sintering (SLS) as the means of scaffold fabrication. Biopolymer PCL and biocomposite PCL/HA were used as the scaffold materials in the validation.

The key contributions are as follows:

- The derivation of relationships between scaffold structure, porosity and compressive stiffness for polyhedral CASTS scaffolds fabricated using SLS has been accomplished. Based on the modified rule of mixtures, the compressive stiffness of the CASTS scaffolds, both PCL and PCL/HA, can be predicted when the scaffold porosity is known.
- The automated system for designing FGS, both radial gradients for long bone applications and linear gradients for short or irregular bone regeneration has been achieved. A complete model relating the FGS parameters to the unit cell dimensions and assembly has also been established.
- *In vitro* studies investigating the effects of varying scaffold porosity on cell proliferation and matrix mineralization have been carried out. The porosity variation yields different values of scaffold surface area, affecting the cell proliferation and matrix mineralization.

These contributions are further discussed in the following sections.

8.1.1. The Effects of Porosity and Structure on Compressive Stiffness and Strength

FGS with stiffness variation mimicking those of target bone can be achieved by varying its porosity with respect to the spatial position as scaffold porosity directly contributes to its compressive stiffness [61]. Using the modified rule of mixtures and compressive testing results of the CASTS scaffolds, the relationships between porosity and compressive stiffness have been established, both for PCL and PCL/HA scaffolds.

In the case of PCL scaffolds, compressive stiffness decreases linearly with increasing scaffold porosity. On the other hand, stiffness increases with PCL/HA volume fraction (1 - porosity) according to power law, which could be due to the stress concentration resulting from the porosity and the unsintered HA particles. These relationships were well predicted using the rule of mixtures ($R^2 > 0.9$).

These relationships are useful in predicting the compressive stiffness of a CASTS scaffold when its porosity and structure are known. This serves as the basis for the development of the automated FGS system discussed in Chapter 4. These relationships have been integrated into the system, allowing user to design either linear or radial bone FGS with desired stiffness gradient.

8.1.2. The Development of FGS Automated System

The success of the CASTS system has motivated this research to take the system to a new level by introducing the FGS automation into the system. The rationale behind this research has been discussed in Chapter 2, in which a successful TE scaffold should be functionally graded to facilitate the appropriately seeded cells to proliferate at the desired layer and to perform their functions properly to achieve the regeneration of healthy tissues.

An automated FGS system has been successfully developed for bone TE based on the stiffness variation possessed by native bones (refer to Chapter 4). The system was

developed in Pro/Engineer environment. Two types of gradients have been generated, namely, the radial and linear/axial gradients. Radial gradient is useful in the case of regenerating a section of long bones such as femur, which consist of cortical part in the outer radius and cancellous part in the inner radius. On the other hand, the gradient in linear direction can be used in generating scaffolds for other types of bone, such as the mandible, and tissue interfaces, such as the bone-cartilage interface.

Using cylindrical unit cells with vertical struts, the model for radial FGS has been established. In this approach, the unit cells with uniform dimensions were assembled in one concentric ring. The rings were assembled concentrically, where different concentric rings possessed unit cells with different dimensions. The dimensions of each cylindrical unit cell were governed by the porosity-compressive stiffness relationships established in Chapter 6. Three main parameters to define and assemble such concentric structure have been established, namely, the cylindrical unit cell size, the radial distance and the radian increment. These concentric ring structures were then stacked to form a concentric cylinder structure.

The shape of the unit cylinder has been designed for flexibility to be assembled into concentric rings, without the need of deforming the structure or adding additional feature to fit them into the concentric ring structure. Therefore, it eases the calculation for scaffold volume, surface area, porosity, and hence its stiffness.

To construct linear FGS, it is proposed that scaffold regions with differing stiffness be positioned side by side. In order to facilitate matching of different regions, the unit cell size was kept constant for all regions, while the strut diameter was varied to account for stiffness gradient.

The process flow of designing customized radial/linear FGS from user input to end product was discussed as follows. Briefly, user inputs to the system included the desired stiffness values and the size of each region. The system would then translate the stiffness inputs into appropriate unit cell dimensions through the established relationships. Unit

cells were assembled into the appropriate radial/linear FGS to allow for Boolean operation with the shape of bone defect in order to obtain customized FGS.

The feasibility of the automated FGS system has been successfully verified using case studies of femur and mandible FGS. This radial and linear FGS system is beneficial in aiding tissue scaffold designers in creating scaffolds with gradients mimicking the target organ. With the inputs of the scaffold stiffness variations and dimensions, the system alleviates the tedious drawing routines and the dependency on the user's computer skill.

8.1.3. The Effects of Porosity on Cell Proliferation and ECM Mineralization

Besides affecting the mechanical properties, such as compressive stiffness and strength, varying scaffold porosity influenced cell proliferation and ECM mineralization. It has been found that varying scaffold porosity affected the surface area of the scaffold, i.e. the higher the scaffold porosity, the lower the surface area. This in turn affected the cell proliferation and function. The cell number on the scaffolds corresponded proportionally with the surface area of the scaffold, as well as, the calcium content of the ECM. Last but not least, it has been shown that three-dimensional scaffold structure is a necessity for cell function, such as ECM deposition and mineralization, reflected by the significantly higher calcium deposit-to-cell number ratio of all scaffolds (with 40%, 60% and 80% porosity value) compared to those of two-dimensional polystyrene well.

8.2. Future Work

The recommended future research is as follows:

8.2.1. Explorations on Biomaterials for Cortical Bone Regeneration

This research has emphasized the development of FGS system with stiffness gradient mimicking the target bone. PCL and PCL/HA scaffolds developed in this research have been shown to fulfill the stiffness requirement for cancellous bone. The FGS system can

be further refined to include wider range of materials, especially with mechanical properties sufficient to fulfill the high stiffness requirement of the cortical bone, which is around 17 GPa [193].

HA with stiffness of 154 GPa [155] could contribute to achieve scaffolds with sufficient stiffness for replacing cortical bone. Due to its brittleness, it should be mixed with biopolymers, such as PCL [143], PVA [194] or PLLA [195]. In this research PCL/HA scaffolds have been fabricated with 10 wt% of HA. The HA concentration was selected for the optimal increase of the composite stiffness [143]. Increasing the HA concentration further could not increase the composite stiffness due to the fact that HA particles could not be sintered with the PCL particles as the binder, hence there was only limited HA concentration that can be added into the PCL matrix [143]. However, in the study by Wiria *et al.* [143] as well as in this research, the particle size of the PCL powder used was much larger ($\sim 100\text{ }\mu\text{m}$) than the HA particle size ($\sim 5\text{ }\mu\text{m}$). If the binder particle size is decreased to be comparable to the HA particle size, the surface area for binding the HA particles becomes larger and possibly higher HA concentration can be added into the binder matrix. And not only PCL could be explored, but also other polymers such as PVA and PLLA. This could be one future work to further improve the FGS system.

8.2.2. Further Characterization of CASTS Scaffolds

This study has focused on matching the scaffold compressive stiffness to those of native bone in compressive loading condition. The working windows of the possible scaffold porosity and compressive stiffness, both for PCL and PCL/HA scaffolds, have been established in Section 6.3. Based on the compressive loading, the SLS fabricated CASTS scaffolds have been shown to fulfill the stiffness requirement for cancellous bone. These comparisons were made based on both dry scaffold and dry cancellous bone conditions during compressive testing.

Due to the fact that bone experiences complex loading condition [18], the research can be expanded to further investigate and characterize the mechanical properties of the CASTS

scaffolds, including bending and/or fatigue properties, as well as, the effect of scaffold degradation on its mechanical properties.

8.2.3. Further *In Vitro* and *In Vivo* Studies

The effects of varying scaffold porosity on cell proliferation and ECM mineralization have been investigated on static cell seeding method (refer to Section 7.5). Further *in vitro* studies on dynamic cell seeding using bioreactor can be proposed to tailor and closely control the culture conditions. Cells seeded onto scaffolds can be subjected to medium flow at an appropriate flow rate that mimicked the natural conditions to facilitate nutrients and wastes transport. Furthermore, mechanical forces resulting from the fluid flow are known to direct cell activity and phenotype, as well as enhancing ECM production [196]. The types of bioreactors available include spinner flasks, rotating vessels and direct perfusion bioreactors [197].

Dynamic cell seeding using bioreactor can be followed up with *in vivo* implantation of FGS into animals, targeting the bones that are of similar mechanical properties with the PCL and/or PCL/HA scaffolds. The issues worth investigating include the effect of scaffold porosity on tissue infiltration and growth, as well as, matrix mineralization.

8.3. List of Publications

The publications that have been a result of this work are listed as follows:

8.3.1. Journal Papers

1. Leong KF, Chua CK, Sudarmadji N and Yeong WY, Engineering functionally graded tissue engineering scaffolds. *Journal of the Mechanical Behavior of Biomedical Materials* (2008) 1: p.140-152.

2. Wiria FE, Sudarmadji N, Leong KF, Chua CK, Chng EW and Chan CC, Selective laser sintering adaptation tools for cost effective fabrication of biomedical prototypes. *Rapid Prototyping Journal* (2010) 16(2): p.90-99.
3. Yeong WY, Sudarmadji N, Yu HY, Chua CK, Leong KF, Subbu S Venkatraman, Freddy Boey YC and Tan LP, Porous polycaprolactone scaffold for cardiac tissue engineering fabricated by selective laser sintering. *Acta Biomaterialia* (2010) 6: p.2028–2034.
4. Sudarmadji N, Tan JY, Leong KF, Chua CK and Loh YT, Investigation of the mechanical properties and porosity relationships in SLS-sintered polyhedral for functionally graded scaffolds. Submitted to *Acta Biomaterialia*.
5. Sudarmadji N, Leong KF and Chua CK, The development of automatic system for designing functionally graded tissue engineering scaffolds. To be submitted to *Computer-Aided Design*.

8.3.2. *Conference Papers*

1. Chua CK, Sudarmadji N and Leong KF, Functionally graded scaffolds: the challenges in design and fabrication processes. Presented in The 3rd International Conference on Advanced Research in Virtual and Rapid Prototyping, 24-29 September 2007, Leiria, Portugal.
2. Leong KF, Chua CK, Sudarmadji N and Yeong WY, Engineering functionally graded tissue engineering scaffolds. Presented as a keynote in The 2nd International Conference on the Mechanics of Biomaterials and Tissues, 9-13 December 2007, Hawaii, USA.
3. Sudarmadji N, Wiria FE, Leong KF, Chua CK, Chng EW and Chan CC, Compact adaptation device for selective laser sintering of tissue engineering scaffolds. Presented in The 7th International Conference on Materials Processing for Properties and Performance, 5-7 November 2008, Singapore.
4. Chua CK, Sudarmadji N, Leong KF, Chou SM, Lim SC and Firdaus WM, Process flow for designing functionally graded tissue engineering scaffolds. Presented in

The 4th International Conference on Advanced Research in Virtual and Rapid Prototyping, 6-10 October 2009, Leiria, Portugal.

5. Chua CK, Sudarmadji N, Leong KF, Chou SM, Lim SC and Firdaus WM, Computer aided design of functionally graded tissue engineering scaffolds. Presented in The Chang Gung University-Nanyang Technological University Forum, 19-20 November 2009, Taiwan.

8.3.3. *Book Chapter*

1. Sudarmadji N, Chua CK and Leong KF, The development of Computer Aided System for Tissue Scaffolds (CASTS) system for functionally graded tissue engineering scaffolds. Computer Aided Tissue Engineering, Springer Verlag, Accepted for Publication, 2009.

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Appendix

Computer Aided System for Tissue Scaffolds

The screen prints of scaffold design process using CASTS (from page 49, Figure 3.3).

Stress-strain plots of PCL CASTS scaffolds. Note: C = configuration number.

Stress-strain plots of PCL/HA CASTS scaffolds. Note: C = configuration number.