Developing a novel biocomposite on selective laser sintering for tissue engineering

Florencia Edith Wiria

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**DEVELOPING A NOVEL BIOCOMPOSITE ON
SELECTIVE LASER SINTERING FOR TISSUE
ENGINEERING**

FLORENCIA EDITH WIRIA
SCHOOL OF MECHANICAL & AEROSPACE ENGINEERING
2007

Developing a Novel Biocomposite on Selective Laser Sintering for Tissue Engineering

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School of Mechanical & Aerospace Engineering

A thesis submitted to the Nanyang Technological University
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2007

ABSTRACT

Tissue engineering (TE) is a rapidly developing field in science. It involves biological, medical and engineering expertises. The advancement in TE is likely to promote major improvements in the treatment of a variety of conditions, from those that are life threatening to those limiting patient's ability to enjoy life fully. A current technical and engineering challenge is to provide good scaffolds for TE. Highly porous 3-dimensional scaffolds primarily serve as cell transplant devices to facilitate structural and functional tissue unit formation of the newly transplanted cells. It is desirable for the scaffolds to degrade as the tissues regain their original strength and structure.

Various conventional methods have been investigated to produce scaffolds. However, these approaches are limited in many areas, such as irregularity of pore sizes and the lack of spatial control of the interior architecture. There have been contamination issues raised concerning the use of potentially harmful solvents in the processing chain. Therefore rapid prototyping (RP) is employed to circumvent this problem.

Selective Laser Sintering (SLS) is chosen as the preferred RP method due to its versatility in processing various polymeric materials and good stability of its products. Proprietary SLS materials are non-biocompatible as they were conventionally invented for production of industrial parts. Suitable biomaterial powders that can be processed in SLS without allowing damages on the material properties need to be identified.

SLS uses a laser heat source to fabricate parts. A theoretical study based on heat transfer phenomena during SLS process was carried out. The study identified the significant biomaterial and laser beam properties that influence the sintering result. The material properties were thermal conductivity, thermal diffusivity, surface reflectivity and absorption coefficient. The influential laser beam properties were laser power and scan speed, which were machine parameters that can be controlled by users. The identification of the important parameters has reduced the number of sintering trials needed to obtain favourable sintering results.

Poly(vinyl alcohol) (PVA) and hydroxyapatite (HA) were chosen as elements for the biocomposite. Spray-drying and mechanical mixing methods were investigated to develop the biocomposite. The most prominent sintering results were obtained by mechanical mixing of as-received PVA and HA powders. The exposure of HA particles could be easily identified, which is important to encourage cell proliferation in further *in vitro* studies. An investigation on mechanical mixing process of as-received PVA and HA powders has shown that this method could yield homogenous biocomposite powders with good repeatability.

Characterization studies found that chemical composition of PVA as the scaffold matrix was not affected during the sintering process. The sintering process also did not affect both the composition and quantity of PVA and HA in the biocomposite. PVA/HA (5 vol.% HA) scaffolds gave compression stress and compression modulus up to 2.26 ± 0.33 MPa and 8.30 ± 1.1 MPa, respectively. These compressive mechanical properties were suitable for application in the craniomaxillofacial skeleton area.

The simulated body fluid (SBF) study of immersing PVA/HA scaffolds established the significance of HA incorporation in the polymer-based scaffold. Hydroxycarbonate apatite (HCA) layer was found on the scaffold. HCA is similar to the mineral phase of bone chemically and structurally.

Cell culture study using osteoblast-like Saos-2 cells found that cell proliferation was more favourable in a 3-dimensional culture than on a monolayer one. The cells migrated into the inner part of the scaffolds and not just proliferate only on the scaffold surface. It showed that the interconnected porous network achieved by the SLS process was sufficient to induce cell migration, as well as oxygen and nutrient transport, into the inner part of the scaffold. Saos-2 cells were found to prefer PVA/HA scaffolds rather than the pure PVA ones. The incorporation of HA was found to enhance cell attachment, proliferation and distribution of cells in the scaffold.

These favourable findings ascertained the feasibility of PVA/HA biocomposite to be processed in SLS without generating any adverse effect for mammalian cell culture. This

study contributed to the scientific knowledge that sintered synthetic biomaterials, such as PVA, were able to provide a living environment for cells and support the cell growth.

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LIST OF SYMBOLS

$\hat{n}$	Complex refractive index
$\overset{\circ}{E}_g$	Energy generated
$\overset{\circ}{E}_{st}$	Energy storage
∇T	Gradient vector of temperature
q_ϕ''	Heat flux component in azimuthal direction
q_θ''	Heat flux component in polar direction
q_r''	Heat flux component in radial direction
$\mathbf{q}''$	Heat flux vector
$\overset{\circ}{E}_{in}$	Inflow energy
$\overset{\circ}{E}_{out}$	Outflow energy
$\rho c_p \frac{\partial T}{\partial t}$	Rate of change in thermal energy of the medium per unit volume
$\overset{\circ}{q}$	Rate of energy generated of the medium per unit volume
β	Effective absorption coefficient
κ	Extinction index
γ	Incident angle
τ	Laser duration time
ρ	Material density
α	Material thermal diffusivity
ϕ	Referring to azimuthal distance in spherical coordinate system
θ	Referring to polar distance in spherical coordinate system
(x, y)	Instantaneous position of laser beam
(x_o, y_o)	Initial position of laser beam
A_b	Laser beam area
a_o	Initial radius of a particle
C_p	Material specific heat, under constant pressure

D_b	Laser beam diameter
$dx \cdot dy \cdot dz$	Unit volume in Cartesian system
$I(z)$	Light intensity as a function of depth in z-axis
I_0	Initial light intensity
$ierfc$	Integral complementary error function
k	Material thermal conductivity
LP	Laser power
n	Refractive coefficient
$q(x, y, z, t)$	Laser intensity distribution as a function of time and space
q_0	Laser power intensity
r	Referring to radial distance in spherical coordinate system; average particle radius
R	Surface reflectivity
R_2	Second principal radius
SS	Scan speed
T	Instantaneous temperature
T_∞	Surrounding temperature
$T(z, t)$	Temperature at depth, z, and time, t
T_0	Initial part bed temperature
$T_{surface}$	Surface temperature of powder particle
U	Transmitted wave amplitude
U_o	Initial incident wave amplitude
v_x, v_y	Laser beam scan speed in x- and y- directions
w	Laser beam radius
X	Neck radius
z	Depth in z-axis direction of Cartesian coordinate

LIST OF ABBREVIATIONS

3D	3 Dimension
3DP	3-Dimensional Printing
ABS	Acrolonitrile Butadiene Styrene
CAD	Computer Aided Design
CT	Computed Tomography
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
DSC	Differential Scanning Calorimetry
ECACC	European Collection of Cell Culture
FDA	Food and Drug Administration
FDM	Fused Deposition Modelling
FTIR	Fourier Transform Infra Red
HA	Hydroxyapatite
HCA	Hydroxycarbonate Apatite
JCPDS	Joint Committee of Powder Diffraction Standards
MEM	Minimum Essential Media
MM	Model Maker
MRI	Magnetic Resonance Imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide
PBS	Phosphate-Buffered Saline
PCL	Poly- ϵ -Caprolactone
PEEK	Polyetheretherketone
PGA	Poly(Glycolic Acid)
PLA	Poly(Lactic Acid)
PLGA	Poly(Lactic-co-Glycolic Acid)
PLLA	Poly(L-Lactic Acid)
PP	Polypropylene
PVA	Poly(Vinyl Alcohol)
RP	Rapid Prototyping
SBF	Simulated Body Fluid
SD	Standard Deviation

SEM	Scanning Electron Microscope
SLA	StereoLithography Apparatus
SLS	Selective Laser Sintering
STL	STereoLithography
TCP	Tricalcium Phosphate
TE	Tissue Engineering
T_g	Glass transition temperature
TGA	Thermogravimetric Analysis
T_m	Melting temperature
XRD	X-Ray Diffraction

Chapter 1

INTRODUCTION

This chapter provides the background, objective and scope of the thesis. The thesis organisation will also be presented as a brief overview of the content of this thesis.

1.1. Background

Tissue engineering (TE) is an interdisciplinary field that merges many aspects of engineering and life sciences towards the primary understanding of cell functions and the advancement of biological substitutes. It strives to sustain, restore and enhance tissue and organ functions by delivering the necessary cells, scaffolds and biological environments to the damaged or diseased parts [1].

This field is highly investigated essentially due to three main problems. Firstly, there is a high demand in tissue and organ transplantations. Secondly, there is a lack of donors for safe and specific organs. Thirdly, there still exists the possibility of disease transmission between donor's transplanted organs and recipients or immunological rejection problems. Thus having transplants using recipients' own cells would help to alleviate these problems.

TE is likely to promote major advances in the treatment of a variety of conditions, from those that are life threatening to those limiting patient's ability to enjoy life fully. In the United States alone, it is estimated that one in five people reaching the age of 65 would benefit from temporary or permanent organ-replacement therapy during their remaining life span [2]. The growing interests of TE has been further motivated by the potential of customizing tissue implantation for organs such as heart, bone, liver, cartilage, skin, muscles and pancreatic islets [3-6].

There have been a number of favourable outcomes in TE field, as highlighted by the United States Food and Drug Administration (FDA) approval and industry commercialisation of TE products [2, 7, 8]. Several products that have reached the market are coral-derived bone graft material and artificial skin graft. The artificial skin graft has

not only been applied for the main function to treat burns, but it has also been successfully used to heal foot ulcers that develop as a side effect of long-term diabetes [8].

Although the works in TE have been initiated for some time, there are still several major challenges to overcome. A current major technical and engineering challenge is to establish suitable biomaterial for TE scaffolds [9]. In the guided regeneration approach TE scaffold is also commonly implanted without the cells. The scaffold can be designed to incorporate growth factors and thus is used to enhance natural healing of the defects. In the functional TE scaffold approach to engineer new tissues, cells are grown on matrices outside the body. After the cells have fully attached and proliferated on the matrices, the scaffolds are implanted and incorporated into the body [1]. The second scaffold approach is illustrated in Figure 1.

Figure 1. Matrices in terms of 3D scaffolds serve as cell transplant device [1] (Source: Langer, *et al.* Science 260, p. 920-926)

These highly porous 3D scaffolds primarily serve as cell transplant devices. They facilitate structural and functional tissue unit formation of the transplanted cells. This approach has been widely practised based on various biologic observations [1, 10]. Firstly, tissues cannot be implanted in large volumes, as newly implanted cells are nutrient dependent on existing capillary. The cells will not survive if they are located more than a few hundred micrometers from the nearest capillary until angiogenesis occurs. Secondly, cell attachment to the matrix plays an important role in cell division and differentiation. If dissociated cells are placed in mature tissue as a suspension, they will have difficulty finding attachment sites and will not function. This may limit the total number of viable cells.

Therefore 3D scaffolds are necessary to provide temporary mechanical support for new tissue, as well as to guide cell differentiation and tissue assembly [11]. The current challenge lies in the technique to design and produce these scaffolds with the appropriate biomaterials and appropriate micro- and macrostructures that would enable initial cells to attach and proliferate, while at the same time allowing flow of nutrients and waste.

There have been a number of conventional methods used to produce 3D scaffolds, mainly using polymeric biomaterial as the scaffold matrix. The polymers that have been experimented include polyesters, polyurethane, and poly(vinyl alcohol) [12-16]. The methods that have been used include gas foaming, fibre bonding, solvent casting and particulate leaching [13]. In general, moulds are used to shape of the scaffolds, coupled with various techniques to create pores within the moulded materials. In order to make the polymers fit into the mould, usually solvents are used to liquefy the polymers.

Scaffolds processed by conventional methods have produced porous scaffolds with sufficient void volumes and surface area for cell seeding and attachment [12]. These approaches are limited in many ways, such as the irregularity of pore sizes and lack of spatial control of the interior architecture [13], as pore creation and interconnectivity are very process dependent. In addition, there have been issues raised with regards to potentially harmful solvents being used in the processing chain [17]. This leads to the research for better 3D scaffold fabrication techniques [18].

Rapid Prototyping (RP) is an automated computer-controlled manufacturing process that builds prototypes layer-by-layer directly from computer aided design (CAD) data images. Unlike machining processes, such as milling, drilling or turning, which are subtractive in nature, RP systems join together liquid, powder or sheet materials to form prototypes [17]. This technique has been shown to be capable of building complex industrial models with predetermined micro- and macrostructures accurately in a short period of time [19]. Based on these advantages, several RP techniques have been investigated as viable alternatives for achieving extensive and detailed control over scaffold structural design [20].

RP has been used in various medical fields, such as orthopaedics [21] and dentistry [22]. Early works began with construction of surgical planning tools using patients' computed tomography (CT) data to build surgical models [19, 23]. Later on RP was used for fabrication of implants [21, 24-26] and drug delivery devices [27-32]. In recent years, scientists have begun the exploration to produce TE scaffolds using various RP methods [18, 33-35].

One of the most versatile RP systems in terms of material usage and good part stability is Selective Laser Sintering (SLS). This powder based RP system builds 3D objects by means of a carbon dioxide laser to selectively bond powder particles [19]. Prior to laser sintering the powder is heated to its glass-transition temperature. Conventional SLS materials are non-biocompatible as the SLS was initially designed to build industrial prototypes. Therefore, in order to utilize the advantages of SLS for TE scaffolds appliances, there is a need to find suitable biomaterial powders that can be processed in SLS.

A number of industrial polymers, namely polyamide, polycarbonate, nylon and thermoplastic elastomers, have been employed to fabricate different functional prototypes [19, 36, 37]. Parameters of the SLS machine were designed to enable processing of these propriety materials up to 240 °C [38]. Glass transition temperature of polymers in general is below 150 °C and it is still within the SLS capability [39]. This understanding gives the opportunity to investigate the SLS viability to process biomaterial polymers.

Bone defects due to tumour, trauma or congenital causes may easily reduce its role as the protector of softer tissues or organs. These defects are significant if they appear in cranio- and maxillofacial areas, as they may also affect patients' conditions physically, psychologically and aesthetically. Fortunately, bony tissue has been found to possess the ability to grow outside the normal skeleton by implanting osteogenic materials [40]. This has encouraged studies focusing on bone replacement.

A number of biopolymers have been investigated for bone replacement scaffold development. However, the optimal scaffold matrix material for bone tissue engineering has not yet been developed [41].

Several biopolymers that have been extensively used for tissue engineering scaffold fabrication in general are in the polyester family, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and poly(lactic-co-glycolic) acid (PLGA), and poly- ϵ -caprolactone (PCL) [12]. There have been mixed reactions regarding the biocompatibility of these polymers. Many studies reported that they are sufficiently biocompatible although other studies suggested otherwise [42]. Degradation products of these polymers are acidic in nature [42, 43]. Polyester implants have been reported to solicit adverse responses during tissue repair process, such as raising foreign body response and osteolytic reaction [42]. In addition, the usage of polyester can depress cell adhesion due to their hydrophobic surface [44].

Poly(vinyl alcohol) (PVA) is a biomaterial that has been used for many biomedical and pharmaceutical purposes. Several primary applications of PVA are as an agent in drug delivery system [45, 46], the main substance for soft contact lenses [47] and artificial skin graft and cover for burns and infected wounds [48, 49]. PVA has been approved by the FDA for ocular, auricular, topical and oral administration [50].

PVA is naturally a highly hydrophilic polymer [51]. Hydrophilic surface is preferred in cell-substrate interaction as it promotes cell adhesion, proliferation and differentiation. Being hydrophilic, it also elicits minimal foreign body reaction during implantation [52]. PVA has also been reported to be a blood-compatible material [48]. Degradation of PVA in solid form exclusively consists of water elimination [53], a product that can be easily

adjusted to human body environment. The rate of biodegradation of PVA is ~13% loss of mass after 21 days in liquid culture at room temperature [54]. The breakdown product is acetic acid, which is commonly known as vinegar. It is a weak acid and is non-toxic in a macro scale. PVA biodegradation is not known to cause adverse effect on cells since PVA has been implanted successfully in rabbits [55, 56]. An addition of PVA to PLGA scaffold was reported to be favourable to bone formation when implanted in a rabbit skull [57].

These unique characteristics of PVA as a biomaterial have favoured its use for TE scaffolds. There has been minimal work on scaffold development using PVA as the main material up to the commencement of this project. Hence the investigation of PVA as a potential biomaterial in providing the scaffold matrix that gives the best balance between high porosity and acceptable mechanical strength would assist in extending the knowledge pool of TE scaffold development, especially in the specific area of bone regeneration.

Prior to the advent of TE to aid healing of organ defects, commonly used clinical method to heal bone defect is the use of bone graft. Generally referred to bone derived from the patient or obtained from a donor, bone grafts are implanted to replace the defected bones [58]. Bone graft is still widely used, mainly due to its proven track record of a significant success rate in fixing bone defects. A total knee arthroplasty was shown to be successfully carried out using a tibial cut bone graft [59]. An autologous bone graft has also been demonstrated to aid the healing of an elderly patient's fractured vertebral body without any adverse side effects [60].

As an alternative to heal bone defects, bone TE offers various advantages over bone graft. Firstly, new tissues grown from bone TE approach can have a more similar structures and mechanical properties, if not identical, with the bone at the defect area. Secondly, scaffolds for bone tissue engineering can be easily shaped to fit the defect well, something which can be difficult to do for bone graft [58]. Bone tissue engineering allows the use of bone morphogenic proteins to be added at the site of application [61]. These proteins induce the formation of new bone *in vivo*.

It has been reported that there is no single material that possessed all the properties ideal for TE bone replacement [62]. Present scaffold work in bone TE has shown that biodegradable polymeric material is insufficient strength for load bearing application [63],

although there is a promise for maxillofacial application. For that reason, a solution is to develop composite materials that combine the strength of the parent phases and minimize the undesired characteristics associated with the homogeneous constituents. The term “composite” is reserved to those materials in which the distinct phases are separated on a scale larger than atomic.

Normally the composite properties are significantly altered in comparison to the homogenous constituent materials. In particular for bone repair, composites are employed to augment the overall biological response to the scaffold [62]. Bone, by itself, is one of the many natural biological materials with a composite structure. Other natural composites include wood, dentin, cartilage and skin [64]. In biomaterials it is important that the entire elements of the composite are biocompatible.

Several examples of composites for biomaterial applications are dental fillings [65, 66], ceramic-reinforced poly-methyl methacrylate bone cement, carbon fibre reinforced ultra-high-molecular-weight polyethylene [67] and orthopaedic implants with porous surfaces [65]. Composite also proves to be necessary for general scaffold application to assist reconstruction of multi-tissue organs, tissue interfaces and structural tissues such as cartilage, tendon, ligament and muscle [68].

Recent research for composite fabrication has been focused on developing polymer/ceramic blends [68]. These kind of composite materials improve biocompatibility and hard tissue integration in a way that ceramic particles, which are embedded in the polymer matrix, allow for increased initial flash spread of serum proteins compared to the more hydrophobic polymer surface [17].

This interest has led implants in bone TE to be made of materials that will subsequently allow osseointegration, in which resorbable bone substitute is ultimately replaceable by new bone [66]. Thus, a bone defect will potentially be replaced by natural bone tissue with complete union and full restoration of function without the use of a permanent implant [69].

The two most heavily studied bioceramics are Hydroxyapatite (HA) and β -Tricalcium Phosphate (β -TCP). Both materials encourage bone growth, although HA is more osteogenic than β -TCP [69].

The improved osteogenic ability of HA is attributed to its similarity in composition to natural bone [69]. HA crystals are naturally found as the major mineral component that forms the substance of bones [67]. In dentistry and orthopaedic applications, HA has been specifically used as fillers for bone defects and coatings on metallic implants [65, 67].

These characteristics of HA make it favourable to be included as one of the composite elements to formulate TE scaffolds for bone implementation. Apart from providing the bioactivity to the composite, HA addition to polymers has been reported to increase storage modulus, compressive strength and elastic modulus of the biocomposite [70, 71].

The polymer element in the biocomposite is to introduce the ductility and flexibility effect to the scaffolds, as pure HA produces highly brittle parts. In addition, the polymer acts as a binder that entraps HA in its sintered matrices.

In SLS, there are a number of parameters that influence the interaction of biomaterial powders with the laser beam. Available studies predominantly focus on industrial-based materials [72-75]. It is understandable as the SLS was initially invented to fabricate prototypes for industrial purposes. Therefore a study to assess the heat transfer phenomena in SLS and powder thermal properties, especially with regards to biomaterials, would be necessary to better understand the physics.

These factors lay the foundation for the primary motivation of this project: to explore and investigate PVA as a potential material to build biocomposite TE scaffold on the SLS.

1.2. Objectives

The objectives of this project are as follows:

1. To develop a biocomposite of polymeric-ceramic system that is suitable for processing in SLS.
2. To study the thermodynamics phenomenon in SLS and its relationship with powder-baser biomaterials.
3. To investigate and ascertain the feasibility of SLS for TE scaffold fabrication.
4. To investigate the mechanical characteristics of SLS fabricated TE scaffolds for the purpose of comparing the scaffold properties with those of targeted biological bodies.
5. To study the *in vitro* biocompatibility of TE scaffolds fabricated by SLS.

1.3. Scope

The scope of this project is as follows:

1. Understanding the properties of biomaterials related to this project, comprising of hydroxyapatite and selected biopolymers.
2. Understanding basic thermal phenomena that occur in SLS.
3. Developing the methods for the preparations of the biocomposite.
4. Characterizing SLS-fabricated structures using the developed biocomposite of polymer-ceramic biomaterials.
5. Investigating the feasibility of SLS for fabricating TE scaffolds for bone regeneration applications.

1.4. Organisation of Thesis

This thesis begins with the introduction, describing the background of the research and stating the objectives and scope. It is then followed by a chapter on literature review to present related theory, methods and findings of past research.

Chapter 3 presents a study on SLS heat transfer phenomena with relation to powder particles. Afterwards, Chapter 4 elaborates the methodology adopted to prepare and characterise the biocomposite powder. The fabrication and characterisation results of the SLS-fabricated scaffolds are discussed in Chapter 5 and Chapter 6 details the studies on *in vitro* test on the SLS-fabricated scaffolds.

In Chapter 7, conclusions and possible future work on this project will be presented. Finally, a list of publication resulting from this work is provided.

Chapter 2

LITERATURE REVIEW

Tissue engineering (TE) aims to restore structure and function to a defect by using the body's natural healing response in addition to treatment with one or more of three elements, namely cells, signalling molecules and scaffolds [69]. Restoration and therapies of patients' defects using TE approach will be potentially replaced by natural tissue with full restoration of function without the use of any permanent implants [69].

Chapter 2 reviews the literature concerning the scaffold part of TE, which will be closely related to this project. It would begin with the introduction of TE scaffolds: the definition and requirements, followed by scaffold fabrication techniques via both conventional and rapid prototyping (RP) techniques. Biomaterials that have been used for TE scaffold appliances would also be reviewed.

2.1. Tissue Engineering Scaffolds

Tissue engineering (TE) scaffolds are 3D structures that provide mechanical support during tissue regeneration until the new tissues have regained their own strength and function, while the scaffold finally degrades. Scaffolds act as necessary temporary substrates for anchorage-dependent cells, such as osteoblasts [76].

Scaffold-guided tissue regeneration involves the porous scaffolds to be seeded with immature targeted-cells and growth factors that would act as nutrient for the cells. Newly implanted cells are normally lack in vascular systems to allow nutrient and waste flow, hence cell death is at times inevitable without the support of TE scaffolds [1]. Once cells regain their original structure, strength and vascular systems, the cell-scaffold system is implanted back into the patient's body.

Therefore, the requirements for these scaffolds are:

1. Scaffold materials have to be biocompatible and biodegradable, with matching controlled degradation rate of tissue growth [17, 76].
2. Scaffolds should be highly porous, with more than 90% porosity [33, 76, 77].
3. The pores should be interconnected to facilitate cell seeding, growth and migration throughout the scaffolds and also to ensure the ease of transport flow of both nutrient and waste [33, 76, 78]. Channels inside the scaffolds can be designed such that immature cells grow in the desired direction. Interconnected pores would be able to aid the growth of vascularisation system, which is vital for scaffolds in large volume to ensure long-term survival of the cells [79].
4. Mechanical properties of the scaffolds should ideally match those of the tissue implantation site. Scaffolds should maintain sufficient strength during the duration of tissue growth [17, 33].
5. Scaffolds should have appropriate surface chemistry to support cell signalling, attachment and growth [33].
6. Scaffolds must be sterilisable without altering material properties [76].

Many researchers have looked into various ways to fabricate these 3D porous structures. The following sections review both conventional and RP methods of scaffold fabrication.

2.1.1. Conventional Methods for Scaffold Fabrication

Techniques classified under conventional scaffold fabrication methods are those that build the 3D structures without involving any automation process. Scaffolds that are fabricated using these methods are mostly polymer-based [12-14]. Some of the frequently used polymers are poly(lactic acid) (PLA), poly(L-lactic acid) (PLLA) and poly(lactic-co-glycolic acid) (PLGA). Details of these biomaterials are discussed later in section 2.2.

2.1.1.1. Gas Foaming

Yoon, *et al* [80] examined the production of PLGA scaffolds by means of gas foaming and incorporation of salt leaching technique. The process was performed according to the schematic diagram in Figure 2.

Figure 2. Scaffold fabrication chain via gas foaming [80] (Source: Yoon, *et al.* J. Biomed. Mater. Res. 55, p. 401-408)

PLGA pellets were first dissolved in chloroform to make a viscous PLGA gel paste. The paste was then precipitated into ethanol solution mixed with sodium bicarbonate particles. The gel paste mixture was then put in a mould and the ethanol was partially evaporated at room temperature to obtain a semi-solidified polymer/carbonate mass. This mass was then immersed in citric acid aqueous solution to induce gas foaming and salt leaching within the polymer/carbonate matrices, resulting in a porous scaffold structure with average pore size of 200 μm and 90% porosity.

This method allows alteration of scaffold porosity and mechanical strength by controlling the amount of acid-base reaction during gas foaming reaction. However, this process also includes the use of chloroform, which is carcinogenic [81]. Residue of the chloroform could give rise to contamination. Citric acid employed in this technique may also infiltrate into the scaffold and thus causing the scaffold to have too low a pH.

2.1.1.2. Emulsion Freeze-Drying

This scaffold fabrication method consists of creating an emulsion by homogenisation of the polymer solvent and water, followed by rapid cooling of the emulsion to keep it at liquid-state structure. At the end of the process, the solvent is removed by freeze-drying.

Whang, *et al* [82, 83] fabricated PLGA scaffolds according to the schematic diagram shown in Figure 3.

Figure 3. Emulsion freeze-drying process [82] (Source: Whang, *et al.* Polymer 36, p. 837-842)

PLGA was first dissolved in methylene chloride and homogenized to obtain a PLGA emulsion. The homogenized emulsion was then cast onto a mould to get the final scaffold shape and was quenched in liquid nitrogen environment. Once frozen the sample was freeze-dried to remove the solvent.

A limitation in this method is that the scaffolds do not have controlled porosity. Pore size and network interconnectivity is very dependent on the process. Contamination is also an issue raised should the scaffold be implanted *in vitro* and *in vivo*, due to the use of methylene chloride as the solvent. This chemical possesses carcinogenic potential [84] and can leave a residue in the scaffold.

2.1.1.3. Phase Separation

There are three key steps in phase separation process. The first is to subject the materials into a phase separation process, either solid-liquid or liquid-liquid separation. The second is to sublime the solvent and the last is to remove the solvent from the scaffolds.

Zhang, *et al* [85] used this method to fabricate PLGA/HA and PLLA/HA scaffolds, with dioxane as the solvent. The polymer/HA liquid mixture was heated to 50 °C and was rapidly frozen to solidify the solvent and induce solid-liquid phase separation. The solidified mixture was then immersed in liquid nitrogen to obtain a deep frozen state. The frozen mixture was then freeze-dried to remove the solvent.

Other researchers who used this method include Wei, *et al* [86], Guan, *et al* [16] and Chen, *et al* [87]. They fabricated scaffolds made of nano-HA/PLLA, polyurethaneureas and PLLA, respectively.

Scaffolds produced by this method have irregular pore shapes with uncontrollable pore sizes, resulting in irregularity of porous network. Moreover, this method employs toxic solvents, such as dioxane [85], dimethyl sulfoxide [16], dioxane and pyridine [87], which may raise toxicity issues in further implantation study.

2.1.1.4. Solvent-Casting Particulate-Leaching

The solvent-casting particulate-leaching technique principally puts small particles, as the porogen, at the bottom of a mould before casting the solution of scaffold materials into the mould. Upon freezing, the mould is dried under vacuum and the porogen is leached out from the mould to form porous scaffolds. Figure 4 shows the preparation scheme of the process.

Figure 4. Preparation scheme of solvent-casting particulate-leaching technique [88] (Source: Kawanishi, *et al.* Mater. Sci. Eng. C 24, p. 431-435)

Kawanishi, *et al* [88] and Peter, *et al* [89] had prepared PLGA scaffolds and Sato, *et al* [90] with PLLA, using this method and succeeded in obtaining porous structures. Yet, as this method also needs the basic materials to be in a solution form for casting, a solvent is still needed. Furthermore, there is limited interconnected porous network in the scaffolds. Pores shape and size are also limited to the crystal shape and size of the salt.

2.1.1.5. Melt Moulding Particulate Leaching

Se, *et al* [15] explored the possibility of fabricating scaffolds from homogenous mixture of PLGA and PVA. First, a PLGA/PVA blend sheet was prepared. Fine particles of PLGA and PVA were frozen and crushed in liquid nitrogen freezer mill. The PLGA/PVA fine particle blend was put in a mould and compressed thermally, resulting in PLGA/PVA blend sheets. The sheets were put in a different mould and covered with sodium chloride salt particles. The mould was then thermally compressed by applying pressure at a temperature above the melting temperature of both polymers, thus forming a salt-containing disc block. After the block was taken out of the mould it was immersed in water with mild shaking to leach out the salt and form porous scaffolds.

This method has overcome a few of the limitations of conventional technique, such as avoiding organic solvent. Scaffold shape can be easily changed by varying the mould shape. Porosity of the scaffold is controllable by varying the amount of salt used to cover

the sheets during thermal compressing. Nevertheless, the drawback is that since most biopolymers are semi-crystalline, a high temperature is needed to heat the specimen above the melting temperature. This high processing temperature may have adverse effect on the properties of polymers. There is also a risk of leaving residual salt particles inside the scaffolds.

2.1.1.6. Summary of Scaffold Fabrication via Conventional Methods

After reviewing the various conventional scaffold fabrication processes, Table 1 summarizes the critical assessment of each technique.

Although these methods are useful in the successful production of 3D porous structures, overall there are a number of limitations. The most common one is that solvents are necessary in almost all of the techniques to liquefy the polymers to fit into a mould. Even though the process includes a solvent removal step, there still exists the issue of harmful residual solvent trapped in the scaffolds. Secondly, these techniques lack a precise control in determining pore sizes, shape and interconnectivity of the porous network. The pore formation is still highly process dependent rather than design dependent. Thirdly, particularly for techniques involving particulate-leaching steps, there is a risk of inadvertently leaving residual porogen salts inside the scaffolds.

Due to these constraints, researchers have sought other alternatives to fabricate more ideal scaffolds. One of the alternatives is using RP technologies, which will be discussed in section 2.1.2.

Table 1. Summary of conventional scaffold fabrication techniques

<i>Fabrication Methods</i>	<i>Strengths</i>	<i>Weaknesses</i>	<i>References</i>
Gas foaming	<ul style="list-style-type: none"> • Able to vary scaffold porosity. 	<ul style="list-style-type: none"> • Solvent is needed. • Possibility of residual acid used during gas foaming process. 	Yoon, <i>et al.</i> [80]
Emulsion freeze-drying	<ul style="list-style-type: none"> • Able to vary scaffold shape easily 	<ul style="list-style-type: none"> • Solvent is needed. • Limited control of porosity. 	Whang, <i>et al.</i> [82, 83]
Phase separation	<ul style="list-style-type: none"> • Able to incorporate bioactive particles without compromising their properties. 	<ul style="list-style-type: none"> • Solvent is needed. • Irregular pore sizes and shapes. • Limited interconnectivity of porous network. 	Zhang, <i>et al.</i> [85] Wei, <i>et al.</i> [86] Guan, <i>et al.</i> [16] Chen, <i>et al.</i> [87]
Solvent-casting particulate-leaching	<ul style="list-style-type: none"> • Simple. • Able to vary scaffold shape easily. 	<ul style="list-style-type: none"> • Solvent is needed. • Possibility of leaving residual salts in the scaffolds. • Pore size and shape is limited to the size and shape of salt crystal. • Limited pores interconnectivity. 	Kawanishi, <i>et al.</i> [88] Peter, <i>et al.</i> [89] Sato, <i>et al.</i> [90]
Melt-moulding particulate-leaching	<ul style="list-style-type: none"> • Avoid using solvents. • Able to vary scaffold shape easily. • Able to vary scaffold porosity. 	<ul style="list-style-type: none"> • High temperature is necessary and may alter materials' property. • Possibility of leaving residual salts in the scaffolds. 	Se, <i>et al.</i> [15]

2.1.2. Rapid Prototyping Approach to Fabricate Scaffolds

Since the late 1990s, rapid prototyping (RP) has been investigated as an alternative approach to make scaffolds. RP offers the ability to control the mechanical strength of the product and to pre-design interconnected pores of a scaffold. RP is an automated fabrication method that builds 3D structures from their 2D cross sectional area, layer by layer, based on computer representation data. Figure 5 shows the schematic diagram of various RP machines.

This technology is well integrated with advances in computer aided design (CAD) and image acquisition systems, such as computed tomography (CT) and magnetic resonance imaging (MRI), to obtain digital closed-volume models. CAD data is then converted into .STL (STereoLithography) format, which is the common file type recognized for almost all RP systems. STL manipulation software, such as MAGICS RP, can be used to check and correct errors in the .STL files. As the file is loaded into the RP system's computer, the 3D models are sliced into 2D cross sectional areas, with thickness ranging from 0.025 to 0.5 mm [19].

This section reviews several RP applications with regards to TE scaffold work.

Figure 5. Schematic diagram of various RP machines: (a) FDM [91], (b) SLA [77], (c) 3D Bioplotting [92], (d) 3D Printer [21] (Sources: Too, *et al.* Int. J. Adv. Manuf. Tech. 19, p. 217-223; Cooke, *et al.* J. Biomed. Mater. Res. 64B, p.65-69; Landers, *et al.* J. Mater. Sci. 37, p. 3107-3116; Curodeau, *et al.* J. Biomed. Mater. Res. – Appl. Biomater. 53, p.525-535)

2.1.2.1. Fused Deposition Modelling (FDM)

Fused deposition modelling (FDM) is a solid-based RP technique [19]. The modelling material comes in coils of filament. The filament is fed through an extrusion head and is heated to semi-liquid state. This semi-liquid material would then be extruded through the FDM head. The head would then move in x- and y-directions to deposit the material onto the building platform according to each layer's cross-sectional image. The building platform moves down with a distance of one layer thickness whenever each cross-sectional image has been made and the extruding process by the FDM head continues. As the air surrounding the head is kept at room temperature, the extruded material solidifies quickly. FDM has two heads, one each for modelling and support materials. Layer thickness may be varied from 0.172 to 0.356 mm with accuracy up to 0.038 mm. Figure 5(a) shows the schematic diagram of a typical FDM machine.

A number of materials have been used in FDM to produce TE scaffolds, such as poly- ϵ -caprolactone (PCL) [93-95], PCL-HA composite [96], polypropylene (PP)-tricalcium phosphate (TCP) composite [97] and acrylonitrile butadiene styrene (ABS) [98]. The range of pore size and porosity given by FDM is reported to be 160-700 μm and 35-77%, respectively, depending on the lay-down pattern [93, 94, 97, 99]. Mechanical properties of the products differ according to the materials used. PCL scaffolds are reported to give compressive stiffness of 4-77 MPa, yield strength of 0.4-3.6 MPa, yield strain 4-28% [93, 94]. PP-TCP scaffolds give compression strength of 12.7 MPa [97].

FDM process offers direct scaffold production with good strength and regularity. However, raw scaffolding materials have to come in filament form, making it necessary to have a pre-processing step to shape the biomaterials into filaments of specific size to match the FDM head extruder. FDM process includes heating of the filaments into molten state before having it solidified, therefore there is a concern that the high heat may trigger a change in the material property.

2.1.2.2. ModelMaker II (MM II)

Another commonly used solid-based RP system for tissue engineering applications is ModelMaker II (MM II). The process uses two ink-jet type print heads, one for depositing a thermoplastic modelling material and the other to deposit supporting wax, which move in X- and Y-directions according to the cross-sectional patterns. Both modelling and support materials are melted prior to deposition and solidification on the modelling platform. After each layer is completed, a rotating cutter removes the excess material on the layer's top surface to ensure smooth and even surface for the next layer [19]. It gives good accuracy of up to 0.025 mm.

MM II is used for indirect TE scaffold fabrication by fabricating the negative mould using ProtoBuild™ material, which is the only building material available for MM II. The mould is then sacrificed when the scaffold biomaterial is cast onto the mould [35, 52, 68, 100-104].

Casting biomaterials used includes collagen type I [35, 100, 102-104], HA [68, 101], collagen/HA composite [35], chitosan [52], PLA and PLGA [68]. Scaffolds obtained from MM II moulds can have reproducible features as small as 200 µm [100]. HA scaffolds are reported to have up to 53% porosity, a 3-4% error in dimensional accuracy and 10-13% shrinkage [101].

Even though original MM II is not able to build scaffold directly due to the limitation of available modelling materials, it has been overcome by using the indirect scaffold fabrication technique. Theoretically this method offers a wide pool of scaffolding materials. However, there is a possibility of chemical interactions between the scaffold and mould materials which are non-biocompatible [105]. Dimensional shrinkage experienced by scaffolds during the drying process is highly significant. Collagen-based scaffolds from freeze-drying are reported to shrink up to 16%, while those produced by critical point drying can shrink up to 80% [100, 103]. The shrinkage effect is difficult to control by the user and it is an unwanted limitation of the indirect method. Another drawback is the increased scaffold fabrication time and processes compared with direct methods [68].

2.1.2.3. Stereolithography Apparatus (SLA)

Stereolithography apparatus (SLA) is a liquid-based RP system. The modelling material is photopolymer resin, which is curable one layer at a time by exposure to electromagnetic radiation usually in ultra violet and visible ranges. Figure 5(b) shows schematic diagram of the SLA machine.

TE scaffold fabrication using SLA has been performed using various methods. Cooke, *et al* [77] chemically-engineered curable biodegradable materials. Biodegradable diethyl fumarate and poly(propylene fumarate) were mixed with a photoinitiator and the polymer was then crosslinked with the use of SLA's UV laser.

Despite the effort to use stereolithography for direct TE scaffold fabrication, the indirect route is more popular among researchers [106-108]. Possibly it is due to the complexity of producing such polymeric curable materials.

Using indirect approach, HA suspension can be cast onto the mould, which is then pyrolysed and sintered to leave the HA construct. HA scaffolds made using this method are reported to have sintering shrinkage of ~26%, dimensional error ~ 2% and porosity in a range of 26-52% [106]. Thermoplastic elastomers, such as poly-4-hydroxybutyrate and polyhydroxyoctanoate, have also been used to make heart valve scaffolds using the indirect route [108]. Stampfl, *et al* [105, 109-111] used in-house water-soluble photopolymers to create the SLA moulds. The water-soluble property is expected to be suitable for fabrication of sacrificial cellular structures with feature resolutions as small as 200 μm [105]. A semi-indirect technique has also been attempted, in which ceramic scaffold material was mixed in the SLA curable monomer. Afterwards the photo-curable material was sintered to obtain ceramic scaffolds [112, 113].

2.1.2.4. 3-Dimensional Bioplotting

3-D bioplotting is an RP method based on dispensing technology. Liquid or paste based plotting material is dispensed into a bath of a viscous medium having a density similar to the plotting material (see Figure 5(c)). The resulting buoyancy force compensates the gravity force, allowing the dispensing of both high and low viscosity materials, thus a variety of materials can be processed, including pastes, reactive resins and hydrogels. The

density and polarity of the viscous plotting medium must be selectively chosen to avoid gravity-included structural collapse and use of support [92].

Works by Landers, *et al* [92, 114-116] on 3D bioplotters have constructed scaffolds of hydrogel materials such as agar, gelatine, alginate and polyurethane materials. Pore size of fibrin/alginate acid scaffolds is reported to be 200-400 μm . Most scaffolds produced by 3D bioplotting are targeted for soft tissues, as a majority of the experimental materials are hydrogels [92, 114, 116].

2.1.2.5. 3-Dimensional Printing (3DP)

3-dimensional printing (3DP) is a powder based RP technology that uses adhesive to bond the powders [19]. An inkjet printing head deposits liquid binder according to each layer's geometrical cross-sectional area to glue the layer of powder on the build platform, causing bonding via infiltration of the binder into the powder. The unbound powders become natural support for the model. Schematic diagram of 3DP process is shown at Figure 5(d).

Researchers have worked on 3DP to fabricate TE scaffolds both in direct [117-124] and indirect [125] manners. Biomaterials that have been used in 3DP for TE scaffolds include PLGA, in various ratios of lactic and glycolic [117-119], cornstarch, dextran and gelatine [120], and HA [121-124]. 3DP scaffolds made of full ceramics have to undergo additional post-processing of sintering to remove the polymeric binder. The accuracy of such scaffolds may be compromised as up to 25% sintering shrinkage was reported [121, 123, 124].

3DP process only comprises of binder droplets to attach powder particles in each and between layers, thus it does not require any heating process. Hence, the concern of material degradation is addressed. The precision of 3DP is limited by the size of the powder particles and binder, which is typically restricted to a few hundred microns [126]. There is no chemical fusion among powder particles, thus strength of models may not be sufficient. As 3DP uses binders to attach the powders, selection of a suitable binder material has to be considered carefully. Selection of toxic organic materials such as chloroform as binder [118, 119] may lead to contamination issues.

2.1.2.6. TheriForm™

TheriForm™ is a powder based RP system with similar working principle to 3DP. It utilizes binder droplets to bond powders of the same layer as well as to the previous layer [19]. The binder is selectively dispensed according to each layer's cross sectional area.

Scaffolds are made porous by incorporating sodium chloride in the polymer powder supply and leaching the chloride out from the final structure to form the desired pores [127, 128]. PLLA scaffolds are reported to have final pore size of 38-150 μm and porosity of 75-90% [128]. Scaffolds made of PLGA/TCP composite are reported to give tensile strength and elastic modulus of 1.6-5.7 and 83-233 MPa, respectively. Its compressive strength is 2.5-13.5 MPa and elastic modulus is 54-233 MPa [127].

The ability of TheriForm™ to produce pure HA scaffolds directly is reported by Roy, *et al* [129]. The result showed weak structures as that there was a lack of attachment between the layers due to the absence of permanent fusion between each layer of HA particles. A post processing step of green part heat treatment was necessary to increase the scaffold strength. The heat treatment burned the binder and sintered the scaffolds to fuse the HA particles.

TheriForm™ technology does not incorporate any thermal processing, thus this process is suitable for thermally sensitive polymers that can degrade easily upon thermal influence. However, current available binders are still toxic. Several works used toxic chemicals such as chloroform as the binder [127, 128] and this may raise toxic issue despite of the post-processing step to clean the scaffolds. Furthermore, as there is no permanent fusion among the powder particles, scaffolds may lack the necessary mechanical strength of the natural tissues.

2.1.2.7. Selective Laser Sintering (SLS)

Selective laser sintering (SLS) is an RP system that makes use of a laser beam to selectively fuse the powder-based material together [19]. This process is done layer by layer until the desired solid form is obtained. The unsintered powders become natural support for overhanging configurations. A schematic diagram of SLS process is shown in Figure 6.

Figure 6. Schematic diagram of SLS process [130] (Source: Tan, *et al.* Biomater. 24, p. 3115-3123)

The SLS is equipped with carbon dioxide (CO₂) laser beam with a wavelength of 10.6 μm . This wavelength is well suited for sintering polymer powders, as polymers depict high absorption at far infrared or long wavelength [72]. Therefore SLS is able to process various commercial industrial-based polymeric materials, namely nylon, polyamides, polycarbonate, and thermoplastic elastomer [19].

Early interest in SLS for medical applications was directed to ceramic implant fabrication [24-26, 131, 132]. A group from The University of Texas, Lee, *et al* [24-26] and Vail *et al* [132], used an intermediate polymeric binder, emulsion poly(methyl methacrylate-co-n-butyl methacrylate), to indirectly sinter in house calcium phosphate powders in SLS. The resulting SLS-fabricated polymer-bound ceramic structure was termed the green part and it was then baked to remove the polymer. Anisotropic shrinkage emerged during the baking process but the strength of the final part was sufficient for low-load bearing implants [24]. Another group, Lorrison, *et al*, used apatite-mullite glass-ceramic in an

indirect and direct sintering process [131]. An acrylic binder was blended with the glass-ceramic powder in the indirect approach. The green part obtained was subsequently baked to remove the binder. The glass-ceramic parts produced exhibited significant signs of brittleness.

Later studies exhibit SLS performance for direct scaffold fabrication using biocompatible but non-bioresorbable or bioinert polymers, such as nylon-6 [133, 134] and polyetheretherketone (PEEK) [130, 135]. These works serve as benchmark for subsequent research on biodegradable materials. Several studies that follow include works using biodegradable materials such as PCL [135-139], PVA [135] and PLLA [135]. The studies show promising results for further direct development of TE scaffolds with SLS, with the ability to laser sinter a high melting point polymer in lower temperature environment.

Many of the abovementioned scaffolds are targeted towards bone TE [135, 137, 138]. However, it would be a highly added advantage for bone TE applications if the material from which the scaffold was made possessed a bioactive nature [140]. Literature states that composites, especially those with a bioactive element, are a better choice in providing the appropriate bone tissue replacement, as bone itself is a composite of organic and inorganic constituents [79, 141, 142].

As SLS completely uses neither binders nor solvents in the process there will not be any issues raised regarding infiltration of any kind of materials inside the fabricated scaffolds. A characteristic of SLS models is that they are strong, as sintering causes bonding and densification of particles in a mass of powders by molecular or atomic attraction [143, 144]. Formation of strong and functional parts is carried out by controlling powder fusion and edge definition [145].

SLS resolution is limited by its laser beam diameter, which is 0.4 mm for Sinterstation 2500 [18], and the particle size of the powder material. Another limitation is regarding the heat employed in the process, material properties with regards to thermal degradation have to be taken into account. Ultra high molecular weight polyethylene has been recorded to degrade during the lasing process [146]. It was also reported that powders employed in SLS should be non-porous, dispersed and highly spherical in shape with small amount of

microstructure variety. Powder trapped inside small hollow parts in the model is another problem commonly encountered for porous scaffolds built by the SLS.

2.1.2.8. Summary of Scaffold Fabrication using Rapid Prototyping Methods

Key advantages of RP includes its ability to build complex functional parts accurately, within a short period of time [19]. It also enables custom design and has the potential to control microstructure and overall shape. As one of the requirements for TE scaffolds is good network interconnectivity, RP is able to overcome the limitations of conventional scaffold processing [18, 33]. Furthermore, RP fabrication process does not require the use of solvents, which has been one of the main restrictions in the conventional techniques. Table 2 summarizes the scaffold fabrication using RP methods.

Table 2. Summary of RP scaffold fabrication techniques

<i>RP Systems</i>	<i>Strengths</i>	<i>Weaknesses</i>	<i>References</i>
Fused Deposition Modelling	<ul style="list-style-type: none"> Produce parts with good strength and regularity 	<ul style="list-style-type: none"> Biomaterials have to be pre-processed into filaments with specific size to match the FDM head. High heat during the process may effect material properties. 	<p>Hutmacher, <i>et al.</i> [93, 94]</p> <p>Cao, <i>et al.</i> [95]</p> <p>Schantz, <i>et al.</i> [96]</p> <p>Kalita, <i>et al.</i> [97]</p> <p>Cai, <i>et al.</i> [98]</p>
Model Maker II	<ul style="list-style-type: none"> Very good resolutions. Can be used to fabricate scaffolds made of a variety of natural polymers by using indirect method. 	<ul style="list-style-type: none"> Uncontrollable dimensional shrinkage, up to 80%, during casting process. Increased fabrication time as indirect route is the only approach to fabricate scaffolds. 	<p>Sachlos, <i>et al.</i> [35, 100, 102]</p> <p>Wilson, <i>et al.</i> [101]</p> <p>Manjubala, <i>et al.</i> [52]</p> <p>Taboas, <i>et al.</i> [68]</p> <p>Yeong, <i>et al.</i> [103, 104]</p>
Stereolithography Apparatus	<ul style="list-style-type: none"> Can be used to fabricate scaffolds made of a variety of materials by using indirect method. 	<ul style="list-style-type: none"> Increased fabrication time as indirect route is the only approach to fabricate scaffolds. Hard to remove moulds made of SLA propriety resin as the material is mostly non-water soluble. 	<p>Cooke, <i>et al.</i> [77]</p> <p>Chu, <i>et al.</i> [106, 107]</p> <p>Sodian, <i>et al.</i> [108]</p> <p>Stampfl, <i>et al.</i> [109-111, 113]</p> <p>Porter, <i>et al.</i> [112]</p>

3D Bioplotting	<ul style="list-style-type: none"> • Suitable for hydrogels materials for soft tissue applications. 	<ul style="list-style-type: none"> • Limited processing materials to those that have similar density to the plotting medium. 	Landers, <i>et al.</i> [92, 114-116]
3D Printing	<ul style="list-style-type: none"> • Suitable for thermally sensitive materials. 	<ul style="list-style-type: none"> • Parts are not sufficiently strong. • Binder materials have to be selected carefully. • Additional steps to remove binder materials are needed if they are potentially toxic. • Limited precision to a few hundred microns. 	Griffith, <i>et al.</i> [117-119] Lam, <i>et al.</i> [120] Chumnanklong, <i>et al.</i> [121] Leukers, <i>et al.</i> [122-124] Lee, <i>et al.</i> [125] Ma, <i>et al.</i> [126]
TheriForm	<ul style="list-style-type: none"> • Suitable for thermally sensitive materials 	<ul style="list-style-type: none"> • Parts are not sufficiently strong. • Binder materials have to be selected carefully. • Additional steps to remove binder materials are needed if they are potentially toxic. 	Sherwood, <i>et al.</i> [127, 128] Roy, <i>et al.</i> [129]
Selective Laser Sintering	<ul style="list-style-type: none"> • Wider variety of materials • No binder or solvents is needed. • Good strength of parts. • Porous structures are obtained. 	<ul style="list-style-type: none"> • Resolutions may not be as good as other RP systems. • May not be suitable for thermally-sensitive materials. • Trapped powder is hard to remove. 	Barlow, <i>et al.</i> [24-26, 132] Tan, <i>et al.</i> [130, 135] Lorrison, <i>et al.</i> [131] Hollister, <i>et al.</i> [133, 134, 136-138] Wiria, <i>et al.</i> [139] Rimell, <i>et al.</i> [146]

RP techniques offer a wide variety of methods, with their own characteristics, strengths and weaknesses. However, when compared to the conventional techniques, there are common advantages [18]. The use of solvents in the process chain can be omitted in almost all RP processes. Using the CAD drawing micro- and macrostructures of the scaffolds can be predetermined. Therefore, the potential of RP technique for TE scaffold development is vast and should be further explored.

SLS in particular has distinct strengths compared to other RP techniques, in that it is able to process a wider range of propriety polymeric-based materials compared to other RP systems [19]. Therefore the search for an appropriate biomaterial for scaffolds can be directed towards the use of polymer-based material as the scaffold matrix. Furthermore, direct scaffold fabrication is possible, hence shortening the production steps and time. Available reports on SLS for implant purposes [24-26, 147] may give the basic understanding of biomaterials processing on SLS for scaffold fabrication. For these reasons, SLS has been chosen as the preferred RP method for scaffold development in this project.

2.2. Biomaterials

Biomaterials are materials that are suitable for introduction into living tissue and come into direct contact with blood, tissue or biological fluid inside living bodies. Biomaterials can be classified into natural and synthetic categories. Natural biomaterials include bone, tooth enamel, collagen and chitin. Examples of synthetic biomaterials are titanium metal part for joint prostheses, PMMA bone cement and synthetic polymers. The basic criteria for synthetic biomaterials are that they must be biocompatible, non-toxic, non-carcinogenic, non-mutagenic, have adequate mechanical properties and are suitable to be sterilized without causing property degradation.

This section reviews selected biomaterials that are related to the objective of developing a biocomposite consisting of polymeric and ceramic materials for the purpose of fabricating TE scaffolds using SLS. The polymer would be used for the scaffold matrix while the ceramic is to be incorporated in the matrix to add bioactivity to the scaffold.

Discussion of the polymers is concentrated towards synthetic polymers as natural ones tend to degrade at relatively low temperature [104]. As SLS involves heat in the fabrication process, natural polymers will not be suitable for this project. In addition, synthetic polymers have several advantages compared to natural ones such as collagen, chitin and chitosan.

Advantages of synthetic polymers are discussed as follows [148]:

- Synthetic polymers are normally inexpensive and can be precisely controlled in material properties and quality. Therefore they can be supplied consistently in large quantities with various techniques [13, 148]. This is unlike natural polymers that must be isolated from plant, animal or human tissues and are typically expensive and suffer from large batch-to-batch variations [149].
- Synthetic polymers can be designed to degrade at a controllable rate rather than the variable rate found with the typically enzymatic degradation of natural polymers [13, 148].
- Synthetic polymers are versatile and the properties such as molecular weight and hydrophobicity / hydrophilicity can be customized to meet the needs of applications [148].

- Synthetic polymers generally elicit lower inflammatory response and lower antigenicity when implanted in the body [148, 149]. This is associated with the characteristics of natural polymers that can rapidly interact with the cells and remodel itself in the body [148].

Among others, disadvantages of synthetic polymers are given as follows:

- They possess less capability in providing natural surface for cell attachment and promoting cell proliferation.
- They lack cell-recognition signals.

Only synthetic polymers are considered for this project for the ease of handling and processing, especially with regards to thermal resistance of SLS process.

Bioceramic forms the other constituent of the biocomposite. The need of a biocomposite for bone scaffold development is addressed by Laurencin and Lu [62], as an ideal bone implant cannot be provided by a single material. Therefore composite materials are developed for bone repair for 3 purposes [62]. First, composite improves mechanical properties of the parent phases by increasing structural strength, wear and long-term performance. Second, composites minimize the shortcomings associated with the parent phases, such as neutralizing the detrimental effects of degradation products. In a composite of β -tricalcium phosphate (β -TCP) and poly(propylene fumarate), the ceramic acts as a buffer to neutralize acidic products from the polymer [89]. Lastly, composites are utilized to augment the overall biological response to the scaffold. Improved mechanical strength and optimized structures ultimately promote tissue formation and faster healing [62].

The direction of biocomposite research for bone TE has been concentrated in polymer/ceramic composite [68]. The ceramic portion is focused on bioactive material that can form continuous interface with surrounding bone tissue [62]. As a biomaterial, ceramic is biocompatible and osteoinductive [69].

Two of the most thoroughly investigated bioceramics are β -TCP and hydroxyapatite (HA) [69]. Both of them are advantageous to promote bone growth. However, HA structure

resembles bone mineral more closely than β -TCP does. Therefore HA is believed to be better suited as a scaffold material for bone regeneration [62, 69].

2.2.1. Hydroxyapatite

Calcium hydroxyapatite (HA) can be referred as all mineral phases of calcified tissue in human bodies [150]. It consists of double salt of tricalcium phosphate and calcium hydroxide and is a complex crystal of calcium phosphate, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. This ceramic has molecular weight of 1004.8, calcium to phosphate ratio of 1.67, and density of 3160 kg/m^3 .

Figure 7. Atomic composition of HA [151] (Source: Young, CNSR Publ. no. 230)

HA is soluble in acidic solution, insoluble in alkaline solution and slightly soluble in distilled water. Solubility in distilled water increases with the addition of electrolytes and changes in the presence of amino acids, enzymes, proteins and other organic compounds. These solubility properties are related with tissue biocompatibility. HA was reported to have neither cytotoxicity nor antigenicity [140].

For the past decades, implants have been directed towards the use of bioactive fixation, in which interfacial bonding between implants and tissue is generated from formation of layers of biologically active material on implant surface [62, 152]. HA is classified as a bioactive material that exhibits high osteoconductivity and bioactivity [140]. Being

osteoconductive, fixation forms a bioactive bond to bone with strength equal to, if not greater than, bone after 3-6 months. This high strength of both hard and soft tissue bonding to bioactive implants originates from *in vivo* growth of a dense layer of hydroxycarbonate apatite (HCA) crystal agglomerates, which bind to collagen fibrils in bone structure [152].

The osteoconductivity of HA can be associated with its effective delivery of transforming growth factor- β (TGF- β), fibronectin and cells to the defect site [69]. TGF- β includes bone morphogenetic protein that induces bone formation *in vivo* at the implantation side, whereas fibronectin promotes cellular adhesion and migration.

In polymeric-based implants HA plays a part in introducing bioactivity and improving mechanical properties of implants. It is reported that compressive strength of pure HA implant from 2.6-5.2 MPa whilst the compressive modulus is 75.5-151.3 MPa [153]. Therefore, merging HA with polymers can result in stronger composite. There is a mixed reaction regarding the issue of stress concentration if the embedded particles are much stiffer and stronger than the matrix [154]. Matrix cracking and particle/matrix interface unbinding may become the major damage modes for these kinds of composite and this may be a trade-off in incorporating HA into the polymer matrix. However, there are research that show otherwise. A stress analysis of HA/PLA composite by Balać, *et al* showed that stress concentration factor decreases with the increase of HA particle volume fraction [154].

HA has been used for many different functions in medical applications, mostly related to orthopaedics. It has been investigated as coating for implants, such as bone cement and hip-joint implants to enhance long-term fixation due to its conductivity [155], as materials for bone and tooth implants [24, 25, 156], or combined with polymers as composites for orthopaedic implants, such as with PEEK [157], chitin [158] and chitosan [140]. There are also reports about HA being used as orthopaedics drug delivery systems [71, 159, 160]. HA has been used for fabricating scaffolds, both in conventional [85] and RP methods [35, 101, 121, 129, 130, 139].

2.2.2. Poly(Vinyl Alcohol)

Poly(vinyl alcohol) (PVA) is a biodegradable and biocompatible semi-crystalline polymer with the following chemical structure [161],

PVA was first prepared by Herrmann and Haehnel in 1924 [161]. The monomer vinyl alcohol cannot be obtained in quantities and purity that makes polymerization to PVA possible. Hence it is commercially produced by polymerization of vinyl acetate to poly(vinyl acetate) followed by conversion of the poly(vinyl acetate) to PVA [161, 162].

The conversion from poly(vinyl acetate) to PVA can be carried out using three methods, namely transesterification, hydrolysis and aminolysis [163]. The most common conversion method is hydrolysis, which occurs according to chemical reaction shown in Figure 8.

Figure 8. Hydrolysis of poly(vinyl acetate) to PVA

The catalyst used in the process can be of either acidic or alkaline nature [163]. The most commonly used alkali catalysts are sodium or potassium hydroxide, methoxide and ethoxide. Acid catalysts used are normally strong mineral acids such as sulphuric or hydrochloric acid. The use of acid catalysts is limited commercially as acid hydrolysis rate is much slower than that of alkaline catalysed reaction. In the hydrolysis process an ester exchange reaction is carried between poly(vinyl acetate) and a primary alcohol, most commonly methanol [163]. This reaction gives a by-product of acetic acid.

Being a semi-crystalline polymer, its melting temperature, T_m , and glass transition temperature, T_g , are dependent on the degree of cross-linking. The range for T_m is 220-240 °C. T_g range is 58-85 °C for partially and fully hydrolyzed PVA but it decreases in the presence of water. This polymer has a density of 1190 kg/m³ and thermal conductivity of ~2 W/m.K. PVA is a water soluble polymer but its solubility depends on the hydrolysis and the degree of polymerisation. Hydrolysis level is the amount of vinyl alcohol functionality. Typically, grades of PVA are available with hydrolysis levels in the range of 87 to 99+%. With increasing degree of hydrolysis its solubility decreases, but generally solubility increases as temperature increases [105, 161, 164]. PVA is also known for its long-term temperature and pH stability [165].

Degradation of PVA as a solid structure consists of water removal [53], a by-product which is harmless to human body environment. In addition, PVA has been reported to be a blood-compatible material [48].

PVA has been applied for various applications in medical fields. Food and Drug Administration (FDA) of the United States has approved its application for ocular, auricular, topical and oral administration [50]. Based on its hydrogel characteristics PVA has been tissue-engineered for heart valves scaffolds [166], soft tissue purposes [55, 56, 161, 167, 168] and drug delivery systems [46, 165, 169]. Being a blood-compatible material, PVA has been applied for artificial skin and kidney [48].

PVA with high degree of hydrolysis is insoluble in water at 37 °C (body temperature). Therefore it is suitable to be used in a scaffold blend as it is not easily leached out into water or cell culture media [170]. As a cell culture matrix, PVA has been reported to be cell-compatible or at least not inhibitory for cell adhesion and growth [170]. Karageorgiou and Kaplan reported that an addition of PVA to PLGA scaffold showed a more favourable result to bone formation compared to a scaffold made of pure PGLA [57].

Manjubala, *et al* reported that hydrophilic surface is advantageous and preferred in cell-substrate interaction as it not only promotes cell adhesion, proliferation and differentiation, but also evokes minimal foreign body reaction on implantation [52]. This information highlights a special characteristic of PVA, that it is highly hydrophilic by nature [51].

Reported compressive and shear modulus of PVA are up to 18 and 0.4 MPa, respectively for a hydrogel formed with 75% water [171]. Tensile modulus and ultimate strength are reported to be up to 830 and 24.1 kPa, respectively, for hydrogels of 30 wt.% PVA [168].

2.2.3. Polyesters: Poly(Lactic Acid), Poly(Glycolic Acid) and Poly(Lactic Glycolic Acid)

Polyesters materials are one of the most common groups of polymers used in TE applications [42, 116], especially the aliphatic polyesters of Poly(lactic acid) (PLA), Poly(glycolic acid) (PGA) and its copolymers. These polyesters are approved by FDA of the United States for human clinical use. The chemical structures of the polyesters are shown in Figure 9.

Figure 9. Chemical structure of: (a) PGA, (b) PLA, (c) PLGA

These materials easily degrade by hydrolysis of the ester bonds, in which the ester linkage is weakened when the materials have reaction with water and thus the whole polymer chain deteriorates gradually. Degradation product of polyesters is resorbable and the degradation rates can be altered by changing the functional groups or polymer architecture [6]. A typical limitation of polyesters is their hydrophobic surface, which can depress cell adhesion. To overcome this deficiency, a mainstream approach is to copolymerization of polyesters with hydrophilic co-monomers such as ethylene glycols and PVA [44, 170].

PGA is highly crystalline (46-50%) polymer and has high strength up to modulus of 7 GPa and tensile strength of 69 MPa. Its recorded T_m is 224-226 °C with T_g of 36 °C. Its degradation product is glycolic acid, which is a natural metabolite. It is reported that at 49 days its crystalline region would degrade with 42% weight loss and complete deterioration

of mechanical properties [42]. With its fast degradation rate, ~6-12 months, this polymer has been used mostly for sutures and short-term fixation.

The PLA chain is more hydrophobic and thus more resistant to hydrolytic attack as compared to PGA. It is soluble in chlorinated-based solvents, which are generally toxic to human. When degradation happens in terms of hydrolytic attack of the ester bonds, PLA gives lactic acid as its by-product, which is normally present in human body.

PLA may appear in its three isomeric forms, i.e. D(-), L(+) and racimic (D,L). PLLA is semi-crystalline with T_m of 173-178 °C with T_g of 60-65 °C. Its reported strength is 2.7 GPa in modulus and it degrades completely in 2 years [42]. In scaffold applications PLLA has been explored both through conventional [90] and RP [128, 135] techniques. PDLLA is amorphous and has T_g of 55-60 °C. Its modulus is 1.9-2 GPa with 12-16 months degradation time.

A drawback of polymers with acidic degradation residue is the possibility of a localized acid concentration increase, which may result in tissue damage due to pH decrease in body fluid. Foreign body responses and osteolytic reactions have been reported when polyesters such as PGA are used as fracture fixation [42]. The same literature also reported that porous PLA-PGA scaffold may promote adverse responses during tissue repair process [42]. As it is, PGA, PLA and their copolymers should be implanted in small quantity. Implants have to be designed to have a constant release of degradation products so that the body has sufficient time to clear the waste products from the implantation site [42].

Poly(lactic-co-glycolic acid) (PLGA) is a copolymer of PLA and PGA. As it is, it has combined properties of both polymers, enabling its degradation time to be tailored. This benefit makes it the most utilized polyester compared to its parent polymers. It is an amorphous polymer with T_g of 45-55 °C. Its reported modulus is 2.0 GP.

PLGA has been used to make porous scaffolds by particulate leaching [88], gas foaming [80], emulsion freeze-drying [82, 83], phase separation [85], solvent casting particulate leaching [88, 89] and melt moulding particulate leaching [15]. By RP methods PLGA has been used to make scaffolds by 3DP [118, 119] and TheriForm [127].

2.2.4. Poly- ϵ -Caprolactone

Poly- ϵ -caprolactone (PCL) is linear aliphatic polyester. It has strength of ~ 0.4 GPa (modulus). Its reported T_m is 58-63 °C. With T_g of -60 °C PCL is rubbery in room temperature. With a degradation time of more than 2 years, PCL is mostly used for implantable long term drug delivery systems [172, 173].

Being an FDA approved biomaterial [174], PCL has been used for many applications. Its applications in TE include artificial skin [175] and scaffolds for bone formation [176-178]. One of its drawbacks is the acidic nature of its degradation product. *In vivo* PCL yields ϵ -hydroxycaproic acid [43].

2.3. Bone

Bones are highly vascularised tissues that have a combination of high compressive and tensile strength due to the composition of HA and collagen. Its primary function is to provide the shape and support for the body, as well as protection for delicate organs. Most bones are made of an outer shell of cortical (compact) bone enclosing a core of trabecular (cancellous, spongy) bone. An adult skeleton typically contains 80% cortical and 20% trabecular bone [69]. Figure 10 shows a typical bone structure.

Figure 10. Bone structure [179, 180] (Sources: Mayer, *et al.* Biodegradable Bone Fixation Devices; Spence. Basic Medical Anatomy)

Bone matrix generally consists of three major components [181, 182]. The first component is inorganic substances, taking up 65-70% of total dry bone weight. HA takes up the most element of this inorganic portion. The second is organic material, taking up 30% of the

whole bone content, in which collagen consumes almost 95% of this amount. The rest of the bone composition is water.

An interesting fact of bony tissue is that it can be made to develop outside the normal skeleton by implanting osteogenic materials [40]. This encourages bone tissues development using scaffolds. Optimal pore size for bone growth *in vivo* is reported to be ~100-400 μm for encouraging attachment, migration and proliferation [82, 183]. However there is also a report stating that significant bone regeneration occurs at larger pore sizes, ranging from 300-1200 μm , with no pore size being statistically different [136].

Integration of bone tissue onto bioactive materials is due to biomineralization of a thin layer of calcium phosphate at the interface between the ceramics and host bony tissue [184]. Assessments in animal models imply that after implantation this layer of calcium phosphate is formed on the bioactive surface at an early period. Thereafter it mediates the integration of bone matrix to metabolize as a part of tissue.

As HA takes the majority of bone component materials, synthetic HA has been heavily studied for integration into bone implant materials. For hip implants HA is sprayed onto the metallic implant surface to enhance bone-implant attachment [155]. In this manner HA is incorporated into the scaffold matrices for bone scaffolds. There have been literatures showing bone growth enhancement when HA is put into the scaffold matrices [140, 185, 186].

Mechanical load requirement in craniomaxillofacial areas is relatively lower than that typically expected of long bones in other body parts. Human cortical femur (thigh bone) and tibia (shin bone) normally have elastic modulus of 9.6-17.4 GPa, tensile ultimate stress of 121-182 MPa and compressive ultimate stress of 133-195 MPa [40]. These values are much higher than the reported mechanical properties of human mandible (lower jaw) bones [187, 188]. Table 3 shows the mechanical properties of bones in human mandible [187, 188].

Table 3. Mechanical properties of bones in human mandible

<i>Bone Type</i>	<i>Property</i>	<i>Strength</i>	<i>Reference</i>
Trabecular bone	Young's modulus (compressive)		Misch, <i>et al.</i> [187]
	<ul style="list-style-type: none"> • With cortical plate • Without cortical plate 	24.9 – 24.0 MPa 3.5 – 125.6 MPa	
Trabecular bone	Ultimate compressive stress	0.22 – 10.44 MPa	Misch, <i>et al.</i> [187]
Cancellous bone of toothless mandible	Young's modulus (compressive)		O'Mahony, <i>et al.</i> [188]
	• Mesio-distal direction	907 ± 849 MPa	
	• Bucco-lingual direction	511 ± 565 MPa	
	• Infero-superior direction	114 ± 78 MPa	

The mechanical properties of the human mandible bones can be used as a feasible target to be achieved from the SLS processed biocomposite when the scaffolds are to be targeted towards cranio-maxillofacial bone areas. A close match in the mechanical properties of implant and targeted bone is desired to prevent both stress shielding [146, 189] and inadequacy of load bearing strength [17].

2.4. Bone Tissue Engineering

Every year millions of people worldwide suffer from unrecoverable bone injuries such as severe bone fractures or bone ablations due to tumour extraction or traumatic injuries [190]. Currently, the most common existing clinical treatment involves placing a bone graft into the defect site, which can be derived from the patients as an autograft bone implant or from a registered bone bank as an allograft bone matrix without cells [58].

There has been a mixed reaction in the scientists' views on the bone replacement method that provides the safest and optimum patient recovery. Autologous bone graft is often considered the most “natural” material of all graft because it possesses good growth potential and resistance to infections. It displays good histocompatibility and bone regeneration process, presents a low incidence of infections, as well as potentially has no foreign body reaction [191]. On the opposite, there are views that harvesting autologous bone graft often adds to the overall time required for surgery. Autologous grafting is often associated with donor site morbidity by ways of infection, chronic pain at the donor site and haematoma formation. Autologous bone graft is also limited in term of minimal supply of suitable bone [58, 190].

Allografts are readily available from tissue bank, but their use carries the risk of disease and pathogen transmission and postoperative complications due to tissue rejection [58, 79]. Bone graft is avascular and it relies on diffusion for survival, therefore the size of the defect and viability of the host bed can limit its use. In large defects the bone graft can often be resorbed before osteogenesis is complete [58].

In the rise of tissue engineering (TE) technology, bone TE seeks to provide a solution which is more favourable than the existing clinical treatments. Bone TE in general also aims to provide a treatment solution which does not require further revision surgeries [58]. A combination of biomaterial scaffolds with biologically active factors is generally used in bone TE [192].

Bone defects in cranial and facial bones are mainly caused by trauma, penetrating head injuries and infected, depressed or open fractures. Other prominent causes are surgical bone demolitions for tumours, infections, congenital cranial and cranio-maxillofacial

anomalies. The primary objective of cranio-maxillofacial reconstructive treatment is to restore the normal barriers protecting the intracranial structures [191].

The reconstructive surgery of the cranio-maxillofacial bones is initially carried out using bone grafts, however the recent research has been directed towards the use of bone TE [192-196]. The critical issues in these reconstructive surgery for bone TE applications include the reconstruction of the complex patient-specific geometry and the suitable biomaterials that would help to promote the new bone formation [69, 192].

An image-based approach using CT or MRI data has been used in the recent development to reconstruct a complex geometry in the shape of cranio-maxillofacial bones. The CT or MRI data provides the scaffold design in the shape of patient-specific geometry. RP technology has also aided in the manufacturing of such scaffolds. Using these combined methods, a scaffold in the shape and size that fits the defect site can be easily obtained in a short period of time [136, 192]. Figure 11 shows an integrated image-based scaffold design process for a minipig mandibular condyle [136].

Figure 11. (a) Integrated image-based scaffold design process for a minipig mandibular condyle, (b) RP-fabricated scaffold [136] (Source: Hollister, *et al.* Orthod. Craniofacial Res. 8, p. 162-173)

Figure 11(a) illustrates the beginning of the imaging process with a CT or MRI scan. A global image-design database is created with surgical attachments. Next, scaffold architecture is designed. The global anatomic and architecture data sets are finally

combined to yield the final design which matches the original defect well. Figure 11(b) shows the minipig mandibular condyle scaffold built directly using an RP system.

A wide variety of materials has been studied for bone TE. These materials include polymers, ceramics and a composite of both polymers and ceramics. The polymers that have frequently been studied include the synthetic polyester family, poly(propylene fumarate), poly(vinyl alcohol) and poly- ϵ -caprolactone and natural polymers such as collagen and hyaluronic acid [69, 79]. Biodegradable polymers are attractive materials for reconstructive as they are greatly versatile.

Inorganic ceramics such as hydroxyapatite (HA) and β -tricalcium phosphate have been used mostly due to their biocompatibility, osteoconductivity and bioactivity natures. They bond chemically to bone when implanted [69, 79, 196]. Composites of biodegradable polymers with inorganic bioactive ceramics have been a particular interest area to engineer scaffolds for bone TE. Apart from having the versatility of the polymers such composites could mimic the properties and morphology of both cortical and cancellous bone [79].

The results of *in vivo* animal implantations have been favourable towards the progress of bone TE. Various types of animal models, such as mice, minipigs and rabbits, have been used to aid the proof of concept of the *in vivo* trials. Williams, *et al* implanted poly- ϵ -caprolactone scaffolds subcutaneously in mice [138]. These scaffolds have been seeded with primary human fibroblast and bone morphogenic protein (BMP)-7. The bone mineral density inside the scaffold pores after 4 weeks was found to lie within the range of normal values measured in human trabecular or cortical bone. In another case of a mouse model, HA, poly(propylene fumarate) and β -tricalcium phosphate scaffolds have been demonstrated to support bone formation [136]. For a larger animal model such as minipigs, HA scaffolds in the shape of the minipig's mandibular defect indicated robust ingrowth after 6 and 18 weeks. Bone was found to occupy 40-50% and 70-80% of available pore volume at 6 and 18 weeks, respectively [136]. A three-fold increase of compressive strength after 9 weeks was observed in porous HA scaffold which was implanted in rabbit femoral condyle [186]. A bonding zone composed of bone-like tissues was found on a HA scaffold at 12 weeks after implantation in rabbits [185]. Enhancement of bone formation was observed when a recombinant of human BMP-2 was given to HA scaffold prior to implantation in rabbit femoral condyle [186].

Current state-of-the-art demonstrates that differences in pore size and scaffold material do not play a significant role in bone tissue regeneration. The addition of growth factors such as BMPs is also displayed to aid in accelerating healing in the animal models. The *in vivo* implantations show that there is significant bone regeneration in both small and large animal models in all pore sizes ranging from 300 to 1200 μm . Bone regeneration was found to occur in a large pool of materials, including HA, poly(propylene fumarate), β -tricalcium phosphate and poly- ϵ -caprolactone [61, 136, 138, 185, 186].

2.5. Summary

From the literature review, it is seen that there is a need for scaffold based tissue engineering. Over the years, the research community has acknowledged the ability of RP techniques to improve TE scaffold fabrication process [18, 20, 33]. Among the many RP processes, SLS is one of the most versatile methods and it allows scaffold fabrication via direct route, which is preferred as it takes shorter time to make scaffold with relatively good stability.

Many biomaterials have been experimented for scaffold fabrication. HA is heavily used for bone applications as it is a natural constituent in human bones. It is bioactive and offers osteoconductivity advantage to the scaffold structure [140]. Pure HA is natively strong yet brittle. Thus, incorporating it with polymer-based biomaterials is a better choice to circumvent its brittleness. PVA is a potential polymer to be combined with HA. It possesses unique characteristics that augment its suitability to be used as a scaffold matrix, such as being blood-compatible and highly hydrophilic [48, 51].

A biocomposite made of these materials, coupled with the advantage of SLS, is believed to have the capability to improve on the currently available TE scaffold. The investigation would be important in furthering a new frontier and to add knowledge to the understanding of SLS-built scaffolds. The study could be directed towards bone TE replacements of defects at the craniomaxillofacial bone area.

Chapter 3

MODELLING OF POWDER PARTICLES HEAT TRANSFER PROCESS IN SELECTIVE LASER SINTERING

This chapter deals with the heat transfer process that is experienced by powder particles in SLS powder bed. The modelling of heat transfer process is conducted to understand the sintering phenomena during SLS process. With the understanding of sintering process through the theoretical modelling, experimental process of biomaterials in SLS could be directed to a certain extent towards the appropriate sintering window so as not to cause unintentional material degradation. The knowledge is essential as biomaterials are not SLS standard materials.

The beginning of this chapter will look into the sintering theory of polymer powder and thermodynamics phenomena in SLS. Subsequent chapters present the study and modelling carried out to understand how the heat transfer phenomena affect the sintering results.

3.1. Theoretical Sintering Process of Polymer Powders

Sintering is a thermal treatment to bond loose particles into a coherent, predominantly solid structure via mass transport events. These mass transports events normally occur at the atomic scale. Particles bond or fuse together when heated to a relatively high temperature, which is normally half of the absolute melting temperature [144]. During sintering particles absorb the energy in the form of heat. Temperature of the particles rises and the particles soften.

A model in Figure 12 describes the sintering stages that two adjacent spherical particles can fully experience [197]. During a significant part of sintering process most of the changes involving viscous flow take place within, and in the vicinity, of the contact zone. Particle penetration, starting from the initial point of contact, is accompanied by neck build-up where the external contour consists of circular arcs, making tangential contact with each other with no sharp corner. In an infinite period of time, the system thus tends to form spherical shapes of minimum surface area, which eventually leads to a single sphere.

Figure 12. Geometrical model describing full sintering steps of adjacent spherical particles

This model explores two distinguishable stages, one when the neck is concave during the early stage neck growth ($X < a_o$), and the other when the neck is convex during the late stage of neck growth ($X > a_o$), where X is the neck radius and a_o is the particle initial radius. The second principal radius, R_2 , initially has a negative value during the concave neck stage. At the end of this stage R_2 goes to infinity where the neck becomes flat ($X = a_o$). In the later stage the neck becomes convex ($X > a_o$) and R_2 decreases positively from infinity to $R_2 = X$ where a single sphere is formed.

In the context of SLS usage to fabricate TE scaffold, it is not necessary for adjacent particles to form a sphere shape as the terminal condition. On the contrary, most formation of the particles stops either at early or late stage of neck growth. This helps to ensure that the fabricated scaffold has sufficient porosity and interconnected pores generated from the SLS process.

3.2. Theoretical Heat Transfer Phenomena at Selective Laser Sintering

In normal sintering in a furnace, heat comes from all direction. Assuming consistent heat is present, powders can be uniformly sintered. The heat source on the SLS is different from that of normal sintering. The laser beam in the SLS is a moving heat source, as illustrated in Figure 13.

Figure 13. Situation of moving heat source in SLS

Heat distribution in SLS can be studied through the optical and thermal behaviour behind the SLS process. Optical behaviour governs the relation between laser light and powder bed surface. Light scattering and absorption by powder bed will be addressed by the optical behaviour. The optical properties of the biomaterial, such as index of refraction, are coupled with properties of the laser beam to estimate the amount of energy absorbed by the powder bed.

Thermal behaviour refers to heat source and heat transfer process in the process bed, including conduction, convection and radiation [198]. Heat transfer happens when there is

a temperature different between two objects, resulting in a transit of energy. Energy is transferred from a place with more energy particles to a place with less energy particles. The physical mechanisms which underlie the heat transfer modes are conduction, convection and radiation [199].

Prior to the laser sintering, the powder is heated up to its glass transition temperature, T_g , on the powder bed. The amount of energy absorbed by the powder is dependent on the energy distribution from the laser beam and thermal properties of the powder.

Fundamental modelling of thermal behaviour, namely convection, conduction and radiation, will be used in this study to describe the physical heat transfer phenomena occurring in SLS powder bed during sintering and derive the governing equations. The system used in this study is assumed to be homogeneous. To simplify the derivation without compromising the accuracy, the powder particles are assumed to have homogeneous properties.

The *Conservation of Energy for a Control Volume* [199] is applied to describe the heat transfer phenomena in SLS and derive the governing equations. A control volume is a fixed region in space bounded by a specific control boundary through which energy and matter may enter or exit. The specific control boundary is also considered as the control surface of the system.

A control volume must have an appropriate time basis for the conservation of energy law to prevail. Within every instant of time, t , there must be a balance between all energy rates, which are measured in Watts (joules per second).

SLS environment can be modelled as a control volume with the periphery of the powder bed as the control surface, as shown in Figure 14. The dashed line denotes the boundary of the control volume.

Figure 14. Control volume of a powder bed

The conservation of energy equation is given in equation (1),

$$\dot{E}_{in} + \dot{E}_g - \dot{E}_{out} = \frac{dE_{st}}{dt} = \dot{E}_{st} \quad (1)$$

The terms used in equation (1) are defined as follows:

- $\dot{E}_{in}$ is the inflow energy, which comes from the laser and heat transfer due to conduction from the surrounding particles adjacent to the control volume.
- $\dot{E}_{out}$ is the outflow energy, which comes from the heat transfer due to conduction, convection and radiation from the powder bed to the atmosphere during cooling when the laser beam is moving to the particles outside the control volume.
- $\dot{E}_g$ is the energy generated, which is the conversion from some other energy form, such as electrical, chemical or electromagnetic. This energy comes from an energy source within the control volume. This term is usually represented using the rate of energy generated of the medium per unit volume, $\dot{q}$. The unit volume is given in Cartesian system as $dx \cdot dy \cdot dz$. The energy generated, $\dot{E}_g$, is expressed as

$$\dot{E}_g = \dot{q} dx dy dz \quad (2)$$

- $\dot{E}_{st}$ is the energy storage, which is related to any changes that happen within the control volume and may be due to changes in the internal, kinetic or potential energies of its content. Energy storage for a 3-D transient case is expressed in terms of time rate

of change of the thermal energy of the medium per unit volume, $\rho c_p \frac{\partial T}{\partial t}$. The energy storage, $\dot{E}_{st}$, is termed as

$$\dot{E}_{st} = \rho c_p \frac{\partial T}{\partial t} dx dy dz \quad (3)$$

(ρ is the material density and c_p is the material specific heat under constant pressure).

For powder particles, the spherical coordinate system is suitable for the description of the conduction heat flux of the control volume (see Figure 15).

Figure 15. Spherical coordinate system

Conduction heat rates can be evaluated from Fourier's law [199]. In spherical coordinates the general form of the heat flux vector, $\mathbf{q}''$, and Fourier's law is

$$\mathbf{q}'' = -k \nabla T = -k \left(\mathbf{i} \frac{\partial T}{\partial r} + \mathbf{j} \frac{1}{r} \frac{\partial T}{\partial \theta} + \mathbf{k} \frac{1}{r \sin \theta} \frac{\partial T}{\partial \phi} \right) \quad (4)$$

where

$$\begin{aligned} q_r'' &= -k \frac{\partial T}{\partial r} \\ q_\theta'' &= -\frac{k}{r} \frac{\partial T}{\partial \theta} \\ q_\phi'' &= -\frac{k}{r \sin \theta} \frac{\partial T}{\partial \phi} \end{aligned} \quad (5)$$

are heat flux components in radial (r), polar (θ) and azimuthal (ϕ) directions, respectively. The term k is the material thermal conductivity.

Applying an energy balance to the control volume in Figure 15, the general form of the heat equation for such a system [199] is given as

$$\frac{1}{r^2} \frac{\partial}{\partial r} \left(kr^2 \frac{\partial T}{\partial r} \right) + \frac{1}{r^2 \sin^2 \theta} \frac{\partial}{\partial \phi} \left(k \frac{\partial T}{\partial \phi} \right) + \frac{1}{r^2 \sin^2 \theta} \frac{\partial}{\partial \theta} \left(k \sin \theta \frac{\partial T}{\partial \theta} \right) + \dot{q} = \rho c_p \frac{\partial T}{\partial t} \quad (6)$$

The assumptions and conditions for heat transfer process in an SLS part bed are:

1. Build process in SLS does not involve any kind of energy conversion. The energy generated term, $\dot{E}_g$, is derived from the energy given by the laser beam. The energy generated, $\dot{q}$, is a heat source acquired from the energy distribution of the laser.
2. Thermal diffusivity, α , of polymer powder is poor, hence 1D approximation to predict heat distribution is sufficient. It would only differ by 5% from the 3D calculation [198]. The change of temperature in each powder particle is only across its radius, r .
3. Convection in the SLS build chamber is a natural convection, which is very small and thus it is negligible.
4. Diameter of laser beam is 400 μm whereas in general the diameter of powder particles is the range of 10-200 μm . Therefore when the laser beam is directly focused onto a powder particle, temperature of the powder particle surface, T_{surface} , is approximately equal to its surrounding temperature, T_∞ ($T_{\text{surface}} \approx T_\infty$). Thus the radiation term is considered negligible.
5. The laser power is constant, as it is set by users and is not changed throughout the sintering process. The laser power is responsible for the initial light intensity, I_0 .

The heat equation then reduces to equation (7) which is specific to SLS environment,

$$\frac{2k}{r} \frac{\partial T}{\partial r} + k \frac{\partial^2 T}{\partial r^2} + \dot{q} = \rho c_p \frac{\partial T}{\partial t} \quad (7)$$

A polymeric powder bed can be treated as a system that has both refraction and extinction properties [200]. Refraction and extinction can be described by considering a coherent plane wave incident on an interior parallel-faced slice of material as in Figure 16.

Figure 16. Incident wave amplitude reduced to transmitted wave by scattering and absorption

Suppose the incident amplitude to be U_0 and that leaving the slice to be U . Some of the incident light is changed in phase in traversing the slice by refraction. The other portion of the light may be lost from the transmitted beam by extinction, in terms of scattering and absorption. The single-headed arrows in Figure 16 represent the absorption and scattering the light, whereas the double-headed ones correspond to the incident and transmitted waves.

Light transmission in materials, such as polymers, can be expressed in terms of complex refraction index [200],

$$\hat{n} = n + \kappa i \quad (8)$$

where

$\hat{n}$ = complex refractive index

n = refractive coefficient

κ = extinction index

The refractive coefficient, n , is responsible for the refraction while the extinction coefficient, κ , is accountable for the extinction portion.

Considering a laser beam shining on the surface of the powder bed, the laser light intensity can be derived from Beer's law,

$$I(z) = (1 - R)I_0 \exp(-\beta z) \quad (9)$$

where

$I(z)$ = light intensity as a function of depth in z-axis

R = surface reflectivity

I_0 = initial light intensity

β = effective absorption coefficient

z = depth in z -axis direction

Effective absorption coefficient, β , and surface reflectivity, R , that govern the light intensity are functions of refraction index of the material at a specific laser wavelength and geometry of the powder particle.

Surface reflectivity, R , is determined by the incident angle of light that travels from a non-absorbing into an absorbing medium [198, 200] (see Figure 17).

Figure 17. Incident angle measured from the surface normal

The refraction index in the non-absorbing medium is given by n_1 , while the absorbing medium has a complex refraction index of $\hat{n}_2 = n_2 + \kappa_2 i$. The incident angle, γ , is measured from the surface normal. In the SLS environment, the absorbing medium is the SLS powder bed while the non-absorbing medium is the atmospheric room where the powder bed is housed.

The effective absorption coefficient, β , can be approximated as the inverse of the particle size [198, 201], with r as the average particle radius,

$$\beta \cong \frac{1}{2r} \quad (10)$$

By taking equations (8)-(10) into account [198, 200], the heat source function for a moving Gaussian laser beam can be derived as follows:

$$q = q(x, y, z, t) = (1 - R) \beta I_0 \exp \left[-\frac{(x - x_0 - v_x t)^2 + (y - y_0 - v_y t)^2}{w^2} - \beta z \right] \quad (11)$$

where

- $q(x, y, z, t)$ = laser intensity distribution
- R = surface reflectivity
- β = effective absorption coefficient
- I_0 = initial light intensity
- (x_0, y_0) = initial position of the laser beam
- (x, y) = instantaneous position of the laser beam
- v_x, v_y = laser beam scan speed in x - and y - directions
- w = laser beam radius
- z = depth in z -axis direction

The heat transfer study in this section theoretically explains the fundamental concept of sintering process in an SLS powder bed. By analyzing equations (7) and (11), it can be seen that both laser beam and material properties contribute to the energy intensity obtained by the powder.

From equation (7), it is seen that the material properties such as thermal conductivity, k , thermal diffusivity, α , and specific heat, c_p , determine the amount of heat conducted from one particle to another. Equation (11) shows that optical properties of polymeric material such as powder reflectivity and effective absorption coefficient also determine the laser intensity distribution. Hence the selection of biopolymer influences the manner in which energy is acquired by the powder bed during the SLS process.

For a moving Gaussian laser beam that is commonly employed in SLS [18, 138], equation (11) illustrates that laser power and scan speed are controllable machine properties that can significantly affect the energy obtained by the polymeric powder. Changes in the laser power would be directly proportional to the changes in laser intensity distribution. On the other hand, changes in scan speed would inversely affect the laser intensity distribution both in quadratic and exponential manners.

3.3. Selective Laser Sintering Temperature Distribution Analysis

The temperature distribution model of an SLS laser beam is illustrated in Figure 18. As the laser beams onto the powder bed, the highest temperature would be at the innermost region, T_1 . That is the spot where the beam directly hits the target, thus it gets the most intense heat. The heat received on that spot would directly sinter the powder particles. The remaining heat is then distributed to the surrounding regions via conduction and the amount of conducted heat reduces as the heat is absorbed to sinter the powder. Hence, the temperature decreases with the increasing distance from the targeted area, so that $T_1 > T_2 > T_3$. The bell-shaped curve is a typical characteristic of a Gaussian distribution.

Figure 18. Temperature distribution of laser beam

The following sections would further model the temperature distribution of the SLS laser beam with respect to a biopolymeric powder. The theoretical heat transfer phenomena developed in section 3.2. is used to model the temperature distribution.

A prediction of temperature distribution with respect to time and sintering depth would be simulated using a commercial programming software. The simulation would be carried out by varying the laser power and keeping the laser scan speed constant. Temperature of

stationary SLS laser beam will be measured using an infrared sensor. The simulation and temperature measurement results would then be compared with actual sintering result. A biopolymer, poly(vinyl alcohol) (PVA), will be used in the SLS processing.

3.3.1. Laser Beam Temperature Distribution Simulation

A temperature distribution simulation was carried out using a one-dimensional heat conduction problem. The powder bed can be represented by a semi-infinite solid with constant thermal properties. Heat is supplied by the laser beam at a constant rate for time duration of τ , after which the supply ceases, resembling to the moving condition of SLS laser beam. The temperature at depth, z , and time, t , is given by Carslaw and Yager [202],

$$T(z, t) = T_0 + 2\sqrt{\alpha} \frac{q_0}{k} \left[\sqrt{t} \operatorname{ierfc} \left(\frac{z}{2\sqrt{\alpha t}} \right) - \sqrt{t - \tau} \operatorname{ierfc} \left(\frac{z}{2\sqrt{\alpha(t - \tau)}} \right) \right] \quad (12)$$

$T(z, t)$ = temperature at depth, z , and time, t

T_0 = initial part bed temperature

α = thermal diffusivity of powder material

k = thermal conductivity of powder material

q_0 = laser power intensity

τ = laser duration time

ierfc = integral complementary error function

Power intensity, q_0 , is obtained from

$$q_0 = \frac{LP}{A_b} \quad (13)$$

with LP = laser power

A_b = laser beam area

Time duration of the laser on the spot, τ , is obtained from,

$$\tau = \frac{D_b}{SS} \quad (14)$$

with SS = scan speed

D_b = laser beam diameter

Poly(vinyl alcohol) (PVA) would be used as the scaffold matrix to be developed on the SLS. Therefore the SLS temperature distribution study is primarily applied to PVA. Physical properties of SLS beam and PVA are given in Table 4.

Table 4. Physical properties of elements in SLS environment

<i>Property</i>	<i>Symbol</i>	<i>Value</i>
Poly(vinyl alcohol) (PVA)		
Specific heat, under constant pressure	C_p	1700 J/kg.K
Thermal conductivity	k	2 W/m.K
Thermal diffusivity	α	$9.26 \times 10^{-7} \text{ m}^2/\text{s}$
Density	ρ	1270 kg/m ³
Complex index of refraction, at 60°C	$\hat{n}_2 = n_2 + \kappa i$	$\hat{n}_2 = 6.37 + 2.31i$
Average powder size (radius)	r	$1.5 \times 10^{-4} \text{ m}$
Extinction coefficient	β	6666.67 /m
Air		
Index of refraction	n_1	1
Laser beam		
Incident angle	γ	0
Maximum beam intensity	I_0	$2.069 \times 10^8 \text{ W/m}^2$
Beam diameter	D_b	$4 \times 10^{-4} \text{ m}$
Beam area	A_b	$1.25 \times 10^{-7} \text{ m}^2$
Surface reflectivity	$R(\gamma)$	0.573

Prediction of the temperature distribution was simulated using MATLAB version 6.5. The laser power used was 3 and 4 W with scan speed was 2032 mm/s, giving laser duration time, τ , of 0.2 ms after which the laser heat supply was ceased. Initial part bed temperature, T_0 , was set at room temperature. The remaining constants were set according to the

physical properties of PVA. The simulation program is attached in Appendix A. The simulation was executed with time periods, t , of 0.25 to 10.25 ms and the results are shown in Figure 19.

Figure 19. Temperature distribution simulation using $\tau = 0.2$ ms, $0.25 < t < 10.25$ ms for laser power:

(a) 3 and (b) 4 W

It is observed from Figure 19 that the temperature drops rapidly after the laser heat supply is taken away, regardless of the laser power used. However, the rapid drop is only observed at the beginning. Afterwards it is seen that there is a slow-down in the rate of temperature drop as the time t increases.

Heat conduction may have caused the slow-down of the rate of temperature drop. As the heat is spread via conduction, it assists to create a more even temperature over the z depth. Therefore as time t increases the temperature gets more even for a prolonged depth.

The sintering depth, z , is observed to increase with the raise in laser power for the same corresponding temperature and time period, t . From equation (12), it is also seen that increasing the initial part bed temperature, T_0 , would also increase the sintering depth, z .

3.3.2. Temperature Measurement of Stationary Laser Beam Verification

The temperature distribution measurement was conducted by using infrared thermography (Avio Thermal Video System, Avio Nippon Avionics). Infrared thermography is capable of capturing the temperature distribution from a heat source in a stationary state. The setup was orientated as depicted in Figure 20.

Figure 20. Temperature distribution measurement setup

Measurement was taken in approximately 1 ms after the stationary laser beam was turned off. This was carried out to prevent excessive energy of the stationary beam for a prolonged period of time from being trapped at the powder bed, which may lead to flames. The laser power was set to 3 and 4 W and measurement results are shown in Figure 21.

Figure 21. Results of infrared temperature measurement of laser power 3 and 4 W: (a) and (c) Temperature distribution, (b) and (d) Gaussian contour of the temperature distribution

Observation from Figure 21(a) and (c) shows that temperature distribution structures of both laser powers are nearly similar, as seen from the shapes of each concentric circle that indicates the respective temperature range. Yet, the temperature difference between the laser powers was significant, even for 1 W difference. This variation was most substantial at the innermost circle, which was exactly where the laser first hit before the heat was distributed to its surroundings. It was noted that the temperature difference may rise up to 20 °C during the measurement.

A closer observation of the temperature map obtained by the infrared thermography reveals subtle differences in the distribution in addition to temperature. Temperature rise from 3 to 4 W was predominantly due to the laser power variations, whereas the differences of the temperature map shape in the innermost region and surroundings were due to the secondary terms of conduction heat transfer across the powder bed. As seen

from Figure 21(b) and (d), the temperature map contour exhibits the Gaussian distribution curve, both in the x- and y-directions of the temperature distribution.

The measurement result is comparable to that obtained from the simulation. The maximum temperature displayed by the measurement for the laser power of 4 W is ~ 95 °C, which was taken in immediately after the beam was turned off and thus the temperature has cooled down rapidly from its initial temperature when the beam was still directed at the targeted spot. This corresponds to the temperature taken at time t between 0.25 to 1.25 ms (see Figure 19).

3.4. Verification of Sintered Polymer Powder in Selective Laser Sintering

PVA powder (99% + hydrolyzed, average molecular weight of 89,000-98,000) obtained from Aldrich Chemical Company was used to for the verification purpose. The powder was sintered using constant scan speed of 1778 mm/s while varying the laser power at 13 and 14 W. The sintering results were observed using a scanning electron microscope (JEOL JSM-5600 LV) and the micrograph images are shown in Figure 22. Statistical measurement was performed to quantify the pore size and neck width. Measurement was taken at random spots of the samples and the results are presented in terms of average $\pm$ standard deviation.

Figure 22. PVA sintered using scan speed 1778 mm/s and laser power: (a) 13 W, (b) 14 W

It can be seen from Figure 22 that there is a substantial difference in the sintering effect, as indicated by the rectangular blocks, by varying the laser power with only 1 W difference.

By referring to Figure 12 regarding the sintering stages of polymer particles, it was observed that at the lower laser power (see Figure 22(a)) the sintering effect has only reached early stage neck growth, indicated by the concave necks formed between adjacent powders. On the contrary, the higher laser power has caused further sintering effect (see Figure 22(b)). The rectangular block shows adjacent powders that had gone into terminal stage of fully coalesced structure. Neck width measurements of scaffolds fabricated using 13 W and 14 W were $78.24\text{ }\mu\text{m} \pm 20.02\text{ }\mu\text{m}$ and $85.55\text{ }\mu\text{m} \pm 44.30\text{ }\mu\text{m}$, respectively (both using $n = 10$).

Pore formation is also controllable by adjusting the parameters. The red circles in Figure 22 indicate the pores in the scaffolds. It can be seen that the pore size in scaffold with the higher laser power is relatively smaller than that formed using the lower laser power. Average pore size formed in samples fabricated using 13 W was $133.04\text{ }\mu\text{m} \pm 24.64\text{ }\mu\text{m}$ ($n = 14$) while samples fabricated using 14 W formed pores with size of $91.06\text{ }\mu\text{m} \pm 17.54\text{ }\mu\text{m}$ ($n = 14$). Analysis with Student's *t*-test at significance level of $p < 0.01$ showed that the difference in pore size is significant.

A physical explanation of why a difference of 1 W could produce such a significant difference in necking size and pore formation can be seen from the pure PVA phase change diagram given by a test using differential scanning calorimeter (DSC). Figure 23 shows a graph obtained from the DSC test.

A 1 W variance in laser power could raise the instantaneous temperature at the sintering spot so high such that the temperature using the lower and higher is causing the polymer to experience different phases. As seen from the DSC diagram in Figure 23 it can be seen that as the temperature increases the total amount of heat flow increases. As such, the polymer experiences different state of phase as the temperature increases. As the temperature is first increased the polymer experiences the glass-transition phase, then melting phase if the temperature keeps increasing. Therefore by varying the laser power just 1 W it is possible for the polymer to be in such different phase depending on the temperature difference resulting from the 1 W variation.

Figure 23. Phase changes of pure PVA

These experimental findings show that with the higher energy intensity obtained by the polymer, the sintering results in the greater neck width but smaller pore size. This provides users with the flexibility of controlling the machine parameters to obtain the desired degree of sintering.

3.5. Summary and Correlation of the Heat Transfer Model of Selective Laser Sintering

The stationary temperature distribution measurements serve as a validation of the Gaussian laser beam physical model illustrated in Figure 18. The Gaussian distribution phenomenon showed by the temperature distribution measurement is caused mainly by conduction of heat from the targeted area to the surrounding regions. This signifies that laser beam heat intensity from the SLS laser beam closely follows this type of distribution. The temperature simulation also shows that with the sintering depth improves with the increase in laser power.

As mentioned previously, powder particles in SLS part bed are sintered using a moving heat source. In a micro scale, the sintering model of a moving laser beam is shown in Figure 24.

Figure 24. Sintering model of a moving laser beam

With a moving laser beam, it is expected that there is an overlap of heat received by powder particles, which would assist in powder sintering process. The overlap is shown in the area shared between the curves when time $t=0$ and $t=t_l$, respectively. As laser scan speed increases, the overlap area reduces. The overlapping area when the beam moves

with scan speed of a is much larger than when scan speed is $(a+x)$, where x is positive. In Figure 24, the areas are shown bounded by solid and dotted lines, respectively.

The sintering model indicates that with increasing scan speed, the amount of heat received by the powder is reduced. This correlation agrees with equation (11), which expresses that the laser intensity distribution decreases as scan speed increases.

Both theoretical simulation and experimental measurement show that part bed temperature, scan speed and laser power are the laser beam parameters that would mainly contribute to sintering quality. By adjusting these machine properties, users have greater control over the desired sintering condition.

Chapter 4

TISSUE ENGINEERING SCAFFOLDS – BIOCOMPOSITE PREPARATION AND CHARACTERIZATION METHODOLOGY

This chapter describes the materials and methods that are used in the preparation of TE scaffold validation. The methods include the preparation of the biocomposite, design, fabrication and characterisation of the scaffold specimens.

4.1. Materials

Pure PVA powder (99% + hydrolyzed, average molecular weight of 89,000-98,000) was obtained from Aldrich Chemical Company, Inc.

There were two types of HA used in this research. The first type was in house HA powder, produced via a spray drying technique, in a manner that will be described in section 4.2. The second was commercially available HA powder sold under the brand name CAMCERAM II HA (Cam Implants BV, Netherlands). This HA powder meets the ASTM F1185 requirements [203] and has a particle size distribution with at least 90 wt.% below 60 μm , as determined by Coulter Counter analysis. The average material density is specified as 3.05 g/cm^3 .

4.2. Preparation of In House Hydroxyapatite Powder

Spray-dried HA was produced in house via direct precipitation reaction of calcium hydroxide (GR for analysis, Merck) and ortho-phosphoric acid 85% (Merck) according to the reaction formula,

The process was carried out by diluting 7M (518.8 g) of $\text{Ca}(\text{OH})_2$ powder with 7 litres of distilled water. The temperature of the $\text{Ca}(\text{OH})_2$ solution was maintained at $40 \pm 5^\circ\text{C}$ and stirred continuously using a stirrer-heater system. Subsequently 4.2M (482.6 g) ortho-

phosphoric acid was diluted into 7 litres of distilled water and dripped into the $\text{Ca}(\text{OH})_2$ solution with a flow rate of 2 litres/hour until acidity of the solution reached a pH of 8.

The HA suspension was then left overnight and spray-dried using a spray dryer (Ohkawara Kakohki Co. Ltd., Model L-12) to obtain the HA powder. In general, 10 litres of HA slurry produces ~350 g of spray-dried HA. The spray dryer was set to the following parameters:

1. Feed rate = 2-3 kg/hour.
2. Pressure drop of hot air filter = 200-300 Pa.
3. Pressure inside drying chamber = -100 to 100 Pa.
4. Inlet temperature = 200 °C.
5. Outlet temperature = 100 °C.

4.3. Preparation of Poly(Vinyl Alcohol) / Hydroxyapatite Biocomposite

The biocomposite of polymer/ceramic was chosen as the material, as the properties of HA (ceramic) are such that by itself it is brittle even though it is tough and is able to give the bioactivity feature to the scaffolds. PVA is expected to impart its hydrophilicity nature to the scaffolds, as hydrophilic surface has been reported to promote cell adhesion, proliferation and differentiation [52]. Thus combining these biomaterials would result in more favourable properties for the scaffolds.

There were three methods investigated for the preparation of the PVA/HA biocomposite, namely spray drying, mechanical mixing and a combination of both.

4.3.1. Spray Drying

With the spray drying process, a biocomposite with the predominant material encapsulating the other was obtained. This process produces a free flowing powder that has agglomerated morphology [24-26]. Spray drying is reported to have the ability to produce spherical particles in the size range of ten to several hundred microns in diameter [204].

In this approach, the composite comprising of 85 volumetric percentage (vol.%) spray-dried HA and 15 vol.% PVA was obtained for laser sintering, in which the spray-dried HA powder was coated with PVA. This process was implemented by diluting in house spray-dried HA powder in distilled water to obtain the spray-dried HA suspension. Subsequently, PVA powder was slowly added to heated distilled water and the solution was continuously stirred to ensure a homogenous solution. The dissolution rate decreased as the concentration of dissolved PVA in the distilled water increased, causing the PVA dissolution to be more difficult. Therefore, greater care had to be put to ensure complete PVA dissolution in the distilled water.

When the PVA granules were completely dissolved, the PVA solution was slowly poured into the pre-prepared spray-dried HA suspension while continuously stirring the suspension. As the presence of PVA in the suspension resulted in a viscous suspension, tending to settle down, the suspension needed to be manually stirred every 15 minutes. In addition, it was noted that for every 9 litres of spray-dried HA/PVA suspension, the yield of spray-drying PVA/HA biocomposite powder was between 150-200 g.

As PVA gave a very viscous suspension, preparation using spray drying method could only make a biocomposite comprising up to 85 vol.% HA and 15 vol.% PVA. Adding more PVA into the solution was not investigated because there was a higher chance the resulting powder would be deposited onto the spray dryer wall and tubing during the process.

4.3.2. Mechanical Mixing

The second method involved mechanical mixing of the powders with no permanent chemical attachment among them. Two types of powder blend were used in this method:

1. Spray-dried-PVA/HA powder, which was made following the previously described methodology, with pure PVA powder.
2. Pure HA powder (CAMCERAM II HA) with pure PVA powder.

The purpose of blending spray-dried-PVA/HA powder with additional pure PVA was to increase the amount of PVA in the mixture. This was in anticipation of the sintering trials of spray-dried-PVA/HA powder which may not yield satisfactory results due to the

smaller quantity of PVA in the composition, as PVA was to function as the polymeric binder during laser sintering. The final proportions for both types of mechanical mixing were approximated to be 95 vol.% PVA / 5 vol.% HA, 90 vol.% PVA / 10 vol.% HA and 85 vol.% PA / 15 vol.% HA.

As PVA had already existed in spray-dried-PVA/HA composite, pure PVA powder was added only until the preferred composition was obtained. To ensure thoroughly mixed powder between PVA and HA, the blend was put on a ball mill mixer (US Stonewear, East Palastine, OH 44413). The rollers were run for 3 hours at 40 rpm. These values were selected, as they were considered sufficient to ensure thorough mixing of the blend [157].

4.4. Selection of Mechanical Mixing Method

A separate independent experiment was carried out to find a suitable mechanical mixing method that gave a homogenous powder blend. Two alternative mixing methods were investigated, namely using a ball mill mixer and a tumbler mixer.

4.4.1. Dry Powder Mixing with Ball Mill Mixer

The first mixing method was to use a ball mill mixer (U.S. Stoneware. East Palastine, OH 44413). A glass bottle coupled with glass ball chargers was used with volume of the balls approximately 50% of the glass bottle volume and the amount of materials made up 25% volume of the glass bottle [205]. The glass bottle was then placed on the mixing roller.

Suitable mixing rate was obtained at approximately 76% of critical rotation rate [206]. At the critical rotation speed, centrifugal force equals gravitational force, thus $2mV^2 = mg$, where m is the mass of the loaded bottle, V is the velocity of the rotating bottle, d is the diameter of the bottle and g is gravitational acceleration, 9.81 m/s^2 . Simplifying this, the optimal rotational rate can now be calculated by equation (16).

$$\text{Optimal rotational speed (RPM), } \omega_{\text{optimal}} = \frac{32}{\sqrt{\text{Outer diameter of bottle}}} \quad (16)$$

Powder mixing on ball milling was performed in a 12cm-diameter bottle with a rotational speed of 92 rpm, as determined from equation (16).

4.4.2. Dry Powder Mixing with Tumbler-Mixer

The second alternative method of powder mixing was by using a tumbler-mixer (Inversina, Bio Engineering AG). The powder mixed was placed in an 8.5 cm diameter polypropylene container, filled with 30% volume of powder. The mixing speed was set at 110 rpm, again based on equation (16).

4.4.3. Powder Grinding

PVA particles were ground to smaller sizes, similar to those of HA, to attain the mixture homogeneity. It has been found that mixing homogeneity is greatly affected by the constituents' particle size [207].

A domestic grinder (Kenwood) was used for this purpose. Heat was generated during grinding due to interparticle friction, thus the PVA powders were kept frozen in a -20 °C freezer to enable prolonged grinding time and reduce the risk of PVA decomposition during grinding. Grinding was done in batches of 100 ml with duration of 5 minutes each batch. Prior to mixing with HA, the ground PVA powder was sieved to 50-100 µm, so as to maintain the size close to HA size range which was 40-70 µm. This PVA size range was chosen as it produced relatively high yield for SLS test sintering.

4.5. Design and Fabrication of Test Specimens

Test specimens were prepared using Pro-ENGINEER Wildfire 2.0. The CAD data of the model was then converted to .STL format before it was sent to the SLS machine for fabrication. The process parameters of SLS were kept at default values except for the part bed temperature, laser power and scan speed.

Process settings for CastForm were chosen as the default values used in SLS (3D Systems Sinterstation 2500). This was due to the following reasons:

1. Compared to other materials in SLS, the default sintering conditions of CastForm are the nearest to those required to sinter PVA. Its default part bed temperature is 50 °C. This value is near to T_g of PVA, which ranges at 32-80 °C obtained earlier from Differential Scanning Calorimeter (refer to Figure 23).
2. Default layer thickness of CastForm, which is 0.1524 mm, is adequately thick for PVA particles. It can therefore ensure enough PVA particles would be used during powder recoating process of each layer.

Single-layer specimens to test sinter the biocomposite were designed to have 0.1524 mm thickness. Test sintering was performed to obtain the most suitable parameters of SLS and composition of biocomposite for successful fabrication results. The part bed temperature was set to 65 °C according to the range obtained from Differential Scanning Calorimeter (DSC) (refer to section 4.8.4. for methodology on DSC), while laser power and scan speed were varied between 3-20 Watts (W) and 889-5080 mm/s, respectively. The intervals were 1 W and 254 mm/s respectively for laser power and scan speed. This range of values is commonly used when ceramic is one of the components being processed [24, 25]. Laser power and scan speed were chosen as the varying parameters as they corresponded with the findings of the theoretical SLS heat transfer modelling discussed in Chapter 3.

4.6. Compression Testing

Compression testing was performed to check the mechanical properties of the scaffolds and to ensure that the properties were according to those of the targeted site. The test was performed with Instron 5569 machine (Instron Calibration Laboratory), with load cell of 50 kN. Samples and testing parameters were prepared and set according to ASTM standard designation D695-02a [208]. The extension speed of the load cell was set at 1 mm/min and each batch of testings had 5 samples.

4.7. Statistical Analysis

Sample mean values and standard deviations were determined for experimentally measured data concerning pore size and sintering necking width. The mean values of measured dimension were compared with a two-tailed Student's t -test at significance level of $p < 0.05$.

4.8. Characterisations

Characterisations of powder and test specimens were carried out by scanning electron microscope (SEM), x-ray diffraction (XRD) spectroscopy, thermogravimetric analysis (TGA), differential scanning calorimeter (DSC) and Fourier transform infrared (FTIR).

4.8.1. Scanning Electron Microscope (SEM)

Test specimens were examined by SEM (JEOL JSM-5600 LV) to analyze the morphological and surface structure. The specimens were gold-coated (Bio-Rad SEM coating system) prior to observations as they were non-metallic.

4.8.2. X-Ray Diffraction Spectroscopy (XRD)

XRD is a versatile and non-destructive technique for identification and quantitative determination of various crystalline forms or compounds present in powder and solid samples. Identification was carried out by comparing x-ray diffractograms between unidentified samples and an internationally recognized database.

Philips PW 1830 XRD was used to characterize all the samples using step scan with the following parametric settings:

1. Angle $2\theta = 10^\circ$ to 80°
2. Step size = 0.02°
3. Time per step = 1 second
4. Generator tension = 40 kV
5. Generator current = 30 mA

The diffractograms obtained were compared with the database maintained by Joint Committee of Powder Diffraction Standards (JCPDS).

4.8.3. Thermogravimetric Analysis (TGA)

TGA is mainly used to determine the decomposition temperatures of a material by heating it up till it burns off and observing its weight loss. Test runs on TGA (Perkin-Elmer Series

TGA 7) were made for each specimen with the weight of each sample taken to be more than 5 mg to ensure that the decomposition temperature could be reached. TGA was also used to ascertain that the PVA/HA mixture was thoroughly mixed and had the desired distribution by taking specimens from various part of the mixture. When the mixture is thoroughly mixed, the degradation rates and percentage weight loss of these specimens should be very similar. TGA for the tests was carried out by heating from room temperature to 800 °C.

4.8.4. Differential Scanning Calorimeter (DSC)

DSC is used to study the changes that have taken place in a material when heated. It includes the thermal transition such as melting of crystalline polymer and glass transition temperature.

Test runs on DSC (TA Instruments DSC 292) for each specimen were conducted with weight of the sample taken to be more than 5 mg, again to ensure its transition temperature could be reached. The heating and cooling temperature ranges were taken with reference to the TGA results. Pure PVA was heated from 0 to 250 °C, using a heating rate of 20 °C/min.

4.8.5. Fourier Transform Infra-Red (FTIR)

FTIR is used to identify the chemical bonds in a material based on their wavelength properties. It gives the chemical bonds of materials, when the bonds vibrate along the operational frequency wavelength. Electromagnetic radiation absorption or reflectance gives signals at frequencies that correlate to the vibration of specific sets of chemical bonds within a molecule. Hence each bond would give signals at different frequency and each distinct molecule would give different overall spectrum.

FTIR (Bio-Rad, Excalibur Series, FTS 3000) using the transmission mode of sample preparation was used to check if the PVA material experience any chemical changes during laser sintering process. Scanning of specimens was performed 4 times, each time from wavenumber 4500 to 400 cm^{-1} . The resolution used was 2 cm^{-1} , with a data interval of 1 cm^{-1} .

4.8.6. Mercury Porosimeter

A mercury porosimeter (Micromeritics, model AutoPore III 9420) was used to measure the porosity of SLS fabricated scaffolds. The pressure range was set to 0 – 241.3 MPa (0 – 35,000 psia) for all measurements.

4.9. Summary

The biocomposite mixture comprised of PVA and HA. Several production methods were investigated. In the first method, a biocomposite powder comprising of in house HA and PVA was obtained using the spray drying technique. The second method was mechanical mixing combining the formerly produced spray-dried-PVA/HA with additional pure PVA powder. The last method was mechanical mixing of pure PVA and HA powder.

A separate independent experiment was conducted to source the most suitable mechanical mixing method that gave a homogenous powder blend. Two alternative mixing methods, specifically using a ball mill mixer and a tumbler mixer, were studied.

Fabrication of test specimens was conducted using process settings for CastForm material. Laser power and scan speed were varied while performing sintering trials to obtain the most suitable SLS parameters.

Scaffold characterizations were assessed by investigating the mechanical properties, porosity, pore size and sintering width. Statistical analysis of sample mean values, standard deviations and Student's *t*-test was conducted to analyse the quantitative results. Material characterizations were performed by using Scanning Electron Microscope (SEM), X-Ray Diffraction (XRD) Spectroscopy, Thermogravimetric Analysis (TGA), Differential Scanning Calorimeter (DSC) and Fourier Transform Infra-Red (FTIR).

Chapter 5

TISSUE ENGINEERING SCAFFOLDS – RESULTS AND DISCUSSION

Chapter 5 presents the results obtained during PVA/HA biocomposite development and sintering trials. Scaffold characterization findings would also be discussed.

5.1. Development of Tissue Engineering Scaffolds of Poly(Vinyl Alcohol) / Hydroxyapatite Biocomposite

This section details the sintering trial results of the biocomposite powders made using various approaches.

5.1.1. Optimisation of Sintering Parameters for Poly(Vinyl Alcohol) Scaffolds

Prior to the test sintering of the PVA/HA biocomposite powder, experiments were carried out with pure PVA powder to establish a set of estimated processing parameters for test sintering the biocomposite powder at the later stage. Table 5 shows the parameters used in laser sintering pure PVA. Single- and 5-layer thickness specimens were built, with a single layer thickness of 0.15 mm.

Table 5. Parameters for laser sintering trials of pure PVA

<i>Part bed temperature</i>	<i>Scan speed</i>	<i>Laser power</i>
60 °C	2540 mm/s (100 in/s) to 5080 mm/s (200 in/s)	3-15 W
65 °C	1270 mm/s (50 in/s) to 1778 mm/s (70 in/s)	

As concluded in Chapter 3, the energy density received by powder is directly proportional to the laser power and inversely proportional to scan speed, to deposit similar or more amount of energy into test specimens, scan speed should be decreased and laser power

increased. The part bed temperature should be increased to provide more initial activation energy prior to laser sintering. When particles have more activation energy, heat from the laser could be more effectively consumed by the powder, thus yielding better sintering results.

Prior to test sintering of pure PVA, it is necessary to check the as-received particle morphology and size. As observed from SEM micrograph shown in Figure 25, the particles have rough shapes with size of approximately 100-200 μm .

Figure 25. As-received PVA powder

Specimens that are considered satisfactory should not be charred and should have structural and handling stability without burning or smoke emission during the processing. During test sintering, specimens may burn if the processing parameters produce excessively high energy density. In order to avoid unnecessary material degradation, the preferred settings for processing were set to a higher scan speed and a lower laser power, without compromising the stability and necking formation. Sintering effect, indicated by the presence of necking among the particles, was observed using SEM.

Initial results using part bed temperature of 60 $^{\circ}\text{C}$ showed that specimens with scan speed of 5080 mm/s had minimal sintering, as sintering effect appeared only at the circumference of the circular disc. These specimens were structurally frail.

Sintering effects started to appear when part bed temperature was increased to 65 °C. Temperature has a most profound influence on the diffusion rate and coefficient. A rise in initial temperature increased the material diffusion coefficient and rate [39], which in turn gave rise to faster collision among molecules in the powder particle. Hence the powder could be sintered more easily. Part bed temperature of 65 °C was then used for subsequent sintering.

Figure 26 shows the favourable sintering window for fabricating satisfactory PVA specimens. The grey area indicates the possible combinations of laser power and scan speed that would yield acceptable specimens for both single- and multiple-layer structures. The allowable scan speed ranges between 1270 to 2540 mm/s while the laser power starts between 10 to 13 W. Areas to the bottom and right of the grey region give no sintering or fragile specimens, while areas to the top and left result in charred specimens.

Figure 26. Sintering window to process pure PVA

Figure 27 shows the SEM micrographs of stable PVA specimens, sintered using various processing parameters within the acceptable sintering window. Sintering signs are apparent at many spots on the specimens. It can be seen that the sintering signs closely follow the geometrical sintering model of spherical particles (refer to Figure 12). The early stage of neck growth is indicated by the arrows. Illustration of particles that have

experienced the late stage of neck growth is highlighted inside the box (see Figure 27(d)), where many particles have finally coalesced to form bigger structures.

Figure 27. Sintered PVA, keywords: scan speed - laser power - magnification: (a) 1270 mm/s - 14 W - 100 times, (b) 2032 mm/s - 13 W - 100 times, (c) 1778 mm/s - 15 W - 25 times, (d) 2540 mm/s - 15 W - 200 times

Sintering neck width measurements of the sintered PVA using various laser powers and scan speeds were analysed. The samples were built by keeping one parameter constant while varying the other. Figure 28 shows the measurement results at random spots ($n = 10$). The measurement shows that an increase in laser power results in thicker neck width. There is a significant difference ($p < 0.05$) among measurements taken every 2 W differences (see Figure 28(a)). Neck width is observed to expand with the reduction in scan speed. A significant difference ($p < 0.05$) is shown for neck width measurement obtained from various scan speeds (see Figure 28(b)).

The neck width measurement results correspond clearly to the theoretical modelling previously described in Chapter 3, section 3.5. The sintering experiment results

demonstrate that sintering quality is significantly improved with the increase in laser power or decrease in scan speed.

Figure 28. Neck width of sintered PVA (n = 10), at: (a) 13 W and various scan speeds, (b) 1270 mm/s and various scan speed. The sign * shows statistical significant difference with $p < 0.05$

Figure 29 shows two of the sintered specimens, built with scan speed of 2032 mm/s and laser power 13 W. The circular disc is 14 mm in diameter and 1.5 mm in height. The rectangular block is a compression test sample with dimension of 12.5 x 12.5 x 25 mm.

Figure 29. Sintered PVA parts: (a) circular disc with 14 mm diameter and 1.5 mm height, (b) compression testing sample 12.5 x 12.5 x 25 mm

5.1.2. Sintering Results of Spray-Dried Poly(Vinyl Alcohol) / Hydroxyapatite Biocomposite

The HA powder used in this method was prepared in-house using a spray-dryer. Figure 30 shows an SEM micrograph of the spray-dried HA powder. The HA particles are spherical in shape with sizes ranging between 40-70 μm . This is consistent with literature report on the spray-drying process being able to produce spherical powder particles [204].

Figure 30. Spray-dried HA powder

Images of spray-dried-PVA/HA are shown in Figure 31. The larger and more spherical particles are identified as PVA particles, whilst the smaller ones are HA.

Figure 31. Spray-dried-PVA/HA biocomposite

The biocomposite, shown by the circles, are formed in spherical shape having sizes of 50-100 μm . It is observed that the spray-drying resulted in HA coated by PVA particles in a random manner. As the composition of PVA and HA is 15:85 (vol.%), it is seen that there are more HA particles than PVA and there are HA particles left either unattached to the PVA particles, shown by the boxes, or stick to the biocomposite particles.

The spray-dried-PVA/HA powder was test sintered according to the parameters shown in Table 6. A total of eight combinations were attempted.

Table 6. Parameters for laser sintering trials of spray-dried-PVA/HA powder

<i>Scan speed</i>	<i>Laser power</i>
1778 mm/s (70 in/s)	10 W
	12 W
1270 mm/s (50 in/s)	14 W
	16 W

Test sintering of this powder did not yield satisfactory results. Test specimens fabricated appeared very fragile as if there were limited fusing between the powder particles. The fragility of the specimens made manual handling of the test specimens almost impossible and examination under SEM revealed little or no necking between the particles. Efforts to further increase the laser power resulted in flaming during the fabrication process, hence making it impossible to fabricate any test specimens. As the relatively low scan speed of 1270 mm/s did not yield any specimens that could be handled, the spray-dried-PVA/HA composite was found to be not feasible for fabrication on the SLS.

The unsuccessful sintering was likely due to the fact that there was too little PVA in the composition, as was the limitation of the spray drying to produce PVA/HA composite. Hence the PVA as a binder could not hold the test specimens strongly together.

Therefore, in order to yield successful sintering, a higher volume percentage of PVA could be used in spray-drying process. However, it should be noted that the spray-drying technique to produce the biocomposite gives rise to three main problems. First, supplying

more PVA into the PVA/HA solution could cause the produced powder to be deposited onto the spray dryer wall and tubing during the process. The second problem was that the powder yield from spray drying did not justify the large amount of slurry that was needed (see Section 4.3.1). The last problem was that with this method it was impossible to distinguish between the PVA and HA particles within the composite powder. Due to these problems, it was decided to mechanically mix the powders before laser sintering.

5.1.3. Sintering Results of Poly(Vinyl Alcohol) / Hydroxyapatite Biocomposite Prepared by Spray-Drying-cum-Mechanical-Mixing

Additional PVA powder is blended with the spray-dried PVA/HA biocomposite via mechanical mixing to add to the PVA content. A series of sintering trials were performed using the parameters detailed in Table 7 for single- and multi-layer specimens. A total of 25 sets experiments were carried out using this type of powder.

Table 7. Parameters for laser sintering trials of spray-dried-PVA/HA (5, 10, 15 vol.% HA)

<i>Scan speed</i>	<i>Laser power</i>
2540 mm/s (100 in/s)	3 – 15 W, with increment of 3 W
2286 mm/s (90 in/s)	
1778 mm/s (70 in/s)	
1270 mm/s (50 in/s)	
889 mm/s (35 in/s)	

Test specimens fabricated with scan speed 889 mm/s were burnt even at low laser power of 6 W although they appeared sufficiently stable. Figure 32 shows the favourable sintering window for spray-dried-PVA/HA (5, 10 and 15 vol.% HA) that yields sufficiently stable specimens. Above and the left hand side areas of the window represent parameters that result in charred or burned specimens. Minimum sintering is obtained when using parameters below and at right hand side of the favourable window.

Figure 32. Sintering window to process spray-dried-PVA/HA (5, 10, 15 vol.% HA)

The experimental results also showed that it was more difficult to sinter the composite with lesser amount of PVA. It is noted from Figure 32 that the smallest favourable sintering window is obtained from the composite with the most HA content. This is likely that with lesser PVA content in the composite, the strength that binds the sintered specimen becomes lesser as well. With lesser PVA there is limited number of neighbouring particles that each PVA particle can form necks with.

Figure 33 shows SEM micrographs of spray-dried-PVA/HA with compositions of 5, 10 and 15 vol.% HA. It was noted that sintering effect occurred both at the top layer of the specimen and also at layers at the lower heights, signified by red and yellow ellipses, respectively (see Figure 33(b)). As laser sintering produces porous specimens, it allows observation of sintering effects of layers beneath the top level.

Figure 33. Sintered spray-dried-PVA/HA at laser power 15 W and scan speeds 1524 and 1778 mm/s, with composition: (a)-(b) 5 vol.% HA, (c)-(d) 10 vol.% HA, (e) 15 vol.% HA

Further observations showed that the necking did not develop into interconnected porous network. Powder particles were fused into lumps of randomly sintered particles. It was also observed that it was difficult to clearly distinguish between the PVA and HA particles. Thus it did not allow the approximation of the amount of PVA and HA particles present in each of the composition by means of visual inspection. This was essential to confirm that the test specimens indeed contained the correct composition of PVA and HA

powder and to ensure that there were HA particles at the scaffold surface, as this was to help cell attachment and proliferation in the scaffold.

To overcome the problems, the spray-dried HA powder was replaced with loose commercial HA powder. The method used to obtain the biocomposite powder was mechanical mixing.

5.1.4. Sintering Results of Poly(Vinyl Alcohol) / Hydroxyapatite Biocomposite Prepared by Mechanical Mixing

Figure 34(a) shows the as-received HA particles used in the mechanical mixing method. Compared to the as-received PVA in Figure 25, HA particles are smoother and smaller in size. It has a dimple-like speck, marked by the yellow arrow, that helps its identification. These differences in as-received PVA and HA would help in distinguishing the particles after sintering. Both PVA and HA particles used are spherical in shape, and this is beneficial as spherical-shape powders allow smooth processing and enhance fluidity of the powder when the SLS roller moves to level the powder.

Figure 34(b)-(d) present the micrographs taken for the three different powder blends containing 5-15 vol.% HA respectively prior to laser sintering. HA particles (as circled) in the three different powder blends were observed to have increasing amounts with increased weight percentage of HA content in the biocomposite. It is worthwhile to note that regardless of the composition, SEM observation of the HA particles on all samples indicates that mixtures with good dispersion and distribution of HA were obtained.

Figure 34. (a) As-received HA used in mechanical mixing and PVA/HA powder blend before sintering in different composition: (b) 5 vol.% HA, (c) 10 vol.% HA, (d) 15 vol.% HA

Acceptable sintering range for spray-dried-PVA/HA (5, 10 and 15 vol.%) were used to assist in designing the sintering trials of PVA/HA processed by mechanical mixing. This measure was to expedite experiments as the composition of both mixtures were similar. Sintering trials were carried out starting from the periphery towards the inner section of the favourable sintering windows. This process was performed for the respective powder composition. Table 8 details the parameters used for sintering trials for the mechanically mixed PVA/HA powder blend for respective composition.

Table 8. Parameters for laser sintering trials of mechanically mixed PVA/HA (5, 10, 15 vol.% HA)

<i>Composition</i>	<i>Scan speed</i>	<i>Laser power</i>
5 vol.% HA	1016 mm/s (40 in/s)	13 W
	1270 mm/s (50 in/s)	5, 13 W
10 vol.% HA	1016 mm/s (40 in/s)	15 W
	1270 mm/s (50 in/s)	7, 13 W
15 vol.% HA	1270 mm/s (50 in/s)	6, 7, 13 W
	2032 mm/s (80 in/s)	10, 15 W

Results of the favourable sintering window result are shown in Figure 35. It can be seen that the shapes of the sintering windows are similar to those of sintered spray-dried-PVA/HA. The smallest window is also occupied by the biocomposite with 15 vol.% HA. This clearly shows that additional HA makes it more difficult to obtain stable specimens as there is less interconnected network formed by the fusion of PVA binder.

Figure 35. Sintering window to process PVA/HA (5, 10, 15 vol.% HA) by mechanical mixing

Figure 36 shows the sintered PVA/HA with the respective acceptable sintering parameters. Prominent sintering effect exhibiting well-defined micro pore interconnectivity can be observed in Figure 36(a), (c)-(d). HA particles could be seen to be well attached onto the sintered network of PVA, as illustrated by the arrows in Figure 36(b). In addition, the HA particles (indicated by circles in Figure 36(a), (c)-(d)) were noted to have been well dispersed in the PVA matrix. It was noted that as the volume percentage of HA in the polymer blend increased, less sintering effects were noted as less PVA particles were fused together. This corresponds to the argument that PVA acts as the polymeric binder in the biocomposite polymer blend and hence explains the difficulty of getting stable specimens for composites with more HA particles. Hence, it is recommended that the volume percentage of HA be kept at 15 vol.% or lower to yield successful scaffold specimens with well-defined pore interconnectivity and good structural integrity.

Figure 36. Sintered PVA/HA prepared by mechanical mixing (keyword: composition - laser power - scan speed): (a)-(b) 5 vol.% HA - 13 W - 2032 mm/s, (c) 10 vol.% HA - 15 W - 1270 mm/s, (d) 15 vol.% HA - 15 W - 1270 mm/s

In addition, HA particles are exposed on the outer surface of the scaffolds. Rizzi *et al* reported that a polymer surface exhibiting exposure of HA were able to provide a novel and favourable substrate for cell attachment [65]. HA structure also resembles bone mineral very closely [69]. Hence the exposure of HA in the PVA matrix would stimulate proliferation of cells, especially bone-related cells, when the scaffolds are used as cell culture substrate.

Mechanical mixing was used as the blending method as it was shown to be able to produce PVA/HA mixture in a relative short period of time (approximately total of 4 hours). The previous method to prepare PVA/HA by spray-drying was inefficient as it took approximately 2-3 days of preparation time for a yield of 150-200 g.

As such, mechanical mixing was chosen as the preferred method to produce PVA/HA biocomposite. Sintering parameters can be chosen to be within the favourable sintering window, with preference to lower laser power and higher scan speed to avoid unnecessary powder degradation. Laser power 13 W and scan speed 2032 mm/s were the parameters used to fabricate samples for scaffold characterizations and cell culture tests. Mercury porosimeter measurement of scaffolds (with 5 vol.% HA) generated 90% porosity. This result is considered sufficiently porous and match the requirement for good TE scaffolds [33, 76, 77]. Pore size at random spots ($n = 34$) of the scaffolds was measured to be $254.07 \mu\text{m} \pm 111.68 \mu\text{m}$. This is within the recommended pore size that encourages bone growth *in vivo* [82, 183].

5.2. Results of the Mechanical Mixing Method Selection

An independent experiment to select the suitable mechanical mixing equipment and method that would give favourable biocomposite powder result was carried out. The powder used for the mixture was as-received PVA and HA powder, consisting of 90 vol.% PVA and 10 vol.% HA.

Two dimensional image characterisations of SEM micrographs were used to visually verify the powder mixing result. Visual inspection of the appearance of mixture and signs of agglomeration in the mixture were also considered. Powder mixture was taken for SEM observation with samples taken at random locations ($n = 3$). Figure 37 shows back-scattered electron (BES) SEM images that were used to ascertain the mixing quality.

Figure 37. Powder blend from various mixing methods: (a) Ball-mill mixing of as-received PVA and HA, (b) Tumbler mixing of as-received PVA and HA, (c) Ball-mill mixing of ground PVA, sized 50-100 μm , and as-received HA. (Note: Darken area showing scale and magnifying degree is not included in the calculation).

Powder sample was taken directly from the mixing container. Particles with brighter contrast and smooth spherical structures were HA, while the ones with darker contrast and rough structures were PVA.

5.2.1. Results of Powder Mixing using Ball Mill Mixer

The first method of mixing is using a ball milling mixer. Upon examination, the ball mill mixture of the as-received PVA and HA appeared homogenous and there were no signs of HA agglomeration as shown in Figure 37(a), which shows one of the SEM micrographs taken from the random locations of the sample.

The amount of HA in the composition was estimated by drawing grids on the BES images (for example shown in Figure 37(a) for ball-mill result), followed by counting the number of grid area taken by the HA. The darkened area of SEM picture showing scale and magnification degree was not included in the calculation as this area was not part of the observation area during the SEM process.

Random spots showed percentage area coverage of the HA to be $9.95 \pm 0.43\%$ ($n = 3$) in the composition. It was very close to the 10 vol.% of the intended mixture and had a reasonably good repeatability.

5.2.2. Results of Powder Mixing using Tumbler Mixer

The next alternate mixing method using a tumbler mixer was investigated. The same ratio of PVA/HA as described in section 5.2.1. was used. Visual observations of the mixing result showed that there were signs of HA agglomeration, mostly located at the bottom of the container. The tumbler mixer works by subjecting the powder container to a 3-dimensional movement (rotation, translation and inversion), resembling an Arabic number eight. The HA powder, being smaller in size and tends to cluster more easily, is thrown by the vigorous shaking movement and thus adheres at the base of the container during the mixing process. This results in poor mixing.

From Figure 37(b), it could be seen that there is a huge concentration of HA particles in some particular area, as highlighted by the circles. Measurement of tumbler-mixed of as-

received powder estimated that the HA particles had taken up $16.67 \pm 3.78\%$ ($n = 3$) of the composition, which was a lot more than the 10 vol.% of the intended composition. This indicates that the HA is not uniformly distributed by this mixing method.

From these analyses, it is deduced that the ball milling mixed is a more effective mixing method to homogenously blend PVA and HA powders. However, the irregularity and larger size of PVA particles, shown by the arrows in Figure 37(a), appears to have blocked the HA particles and this might prevent the HA from being distributed evenly in subsequent repeated process.

By repeating the process with PVA particles of a closer size range of 50-100 μm , a better mixing result was achieved, as shown in Figure 37(c). The percentage area coverage of HA is $9.66 \pm 0.38\%$ ($n = 3$), which is also near to the 10 vol.% of the intended composition but with smaller SD than the mixture of the as-received powders, thus ensuring a higher degree of repeatability. In these analyses, it is deduced that using ball mill mixing while ensuring a regular shape and size of the powder particles can achieve a favourable mixing result that promotes homogeneity.

It was noted that the PVA yield of grinding process was approximately 50% of as-received powder. After sieving to 50-100 μm , the yield was even lesser as compared to the as-received powder. This yield was sufficient for the pilot experiment to achieve the most advantageous mixing. However it may not be feasible to use the smaller sized PVA yield for further experiments, especially since a significant number of samples would be needed for characterization and *in vitro* tests. Hence it was decided to use as-received powders with ball-mill mixing process to produce the PVA/HA biocomposite for further experiments, as this process could obtain homogeneity of components with a good degree of repeatability.

5.3. Characterisation of Poly(Vinyl Alcohol) and Hydroxyapatite

Characterisation of PVA/HA specimens were performed using the mechanically-mixed PVA/HA biocomposite. Three characterisation processes were performed and they included XRD, TGA, compression testing and FTIR.

5.3.1. Chemical Composition Examination of Poly(Vinyl Alcohol)

FTIR was performed to examine the chemical composition of PVA before and after laser sintering treatment. This was to ensure that the PVA did not degrade due to its exposure to heat during the sintering process.

An important characteristic in FTIR analysis is the peak location of chemical bonds along the wavelength. Intensity may vary according to the amount of sample used for analysis.

The basic composition of PVA is $-(CH_2-CHOH)_n-$ and the monomer is $(CH_2=CHOH)$. From this structure, the following functional groups exist: $C-H$, $C-O$, $O-H$ and $C=C$ bonds. FTIR results of the unsintered and sintered PVA samples are plotted and shown in Figure 38, where the peaks are identified according to the type of chemical bonds.

Figure 38. Comparison FTIR spectra of unsintered and sintered PVA

Figure 38 shows that all samples have very similar curves with peak points appearing in the similar wavenumber for each bond. (The wavenumbers, at which the specific bonds appear, can be seen in Appendix B). This indicates that the important bonds of $C-H$, $C-O$, $O-H$ and $C=C$ appear in a similar pattern in the spectra. It is a clear indication that the PVA chemical composition has not been disturbed during the sintering process.

5.3.2. Examination of Hydroxyapatite Existence in Biocomposite Scaffold

X-Ray Diffraction Spectroscopy (XRD) was used to confirm the existence of HA in the biocomposite scaffold. The presence of HA is important to support cell proliferation in *in vitro* experiments.

The material database of Philips PW 1830 XRD used in this examination did not have a reference for PVA. Hence pure PVA was studied under XRD to obtain a diffractogram that would be used as reference. To confirm the presence of a particular material in a

sample, normally 3 highest peaks of the material must be identified. As seen in Figure 39, pure PVA gives the four highest peaks at angle 2θ of 11.46° , 19.62° , 22.52° and 40.58° .

Figure 39. XRD diffractogram of as-received PVA

Figure 40 gives the XRD diffractograms of PVA-HA scaffold with 5 and 10 vol.% HA.

Figure 40. XRD diffractograms of PVA-HA scaffold with 5 and 10 vol.% HA

HA peaks were verified from Joint Committee of Powder Diffraction Standards (JCPDS) reference card no. 9-0432 for calcium phosphate hydroxide. From Figure 40 it can be seen that HA peaks occur at angle 2θ of 25.72° , 32.11° , 32.78° , 46.6° , 49.4° and 72.2° , as indicated by the triangles. These peaks are in accordance with the JCPDS reference, in which the highest peaks occur at angle 2θ of 25.89° , 31.77° , 32.19° , 32.90° , 46.71° , 49.47° and 72.28° .

The PVA at the biocomposite scaffolds are present at the angle 2θ of 11.24° , 19.60° , 22.70° and 40.40° . This occurrence is consistent with the as-received PVA reference in Figure 39.

Subsequent test was Thermogravimetric analysis (TGA), which was performed to verify the amount of HA on the biocomposite scaffold. The analysis was carried out using PVA/HA scaffolds ($n = 5$) with 5 vol.% HA.

In TGA procedure, the processing temperature was increased steadily so that if the degradation temperature of the tested material was within the processing temperature the material would degrade slowly as the heat increases. The TGA recorded the remaining weight of the sample.

In this test, the scaffolds were subjected to temperature above the PVA degradation temperature, but below that of HA. The amount of weight left at the end of the test approximates the relative quantity of HA in the sample.

Figure 41 shows the TGA profile of pure PVA. The profile shows that the PVA can be burnt up by heating it to 800°C .

Figure 41. TGA result of PVA

The sintered PVA/HA scaffolds with 5 vol.% HA (approximately 11.5 weight % HA) was subjected to heat up to 900 °C. The profile is presented in Figure 42.

Figure 42. TGA graph of PVA/HA scaffolds with 5 vol.% HA (n = 5)

As seen from Figure 41 and Figure 42, there is a constant drop of weight from the start of the test until about 100-120 °C, after which the weight remains constant until 230-250 °C. The initial drop is due to moisture being evaporated at about 100 °C. From temperature 230-250 °C, the weight drops drastically until approximately 400-600 °C. This is the range

in which the PVA content is being burnt off, thus causing drastic weight loss. The weight is relatively constant from 600 °C onwards, indicating that all PVA content has been completely degraded.

The experiment showed that the remaining weight % at 900 °C was 11.51 ± 3.78 weight % ($n = 5$). The number is very close to the HA content of the biocomposite powder before sintering.

The XRD and TGA analysis showed that the sintering process did not disturb the composition or the quantity of PVA and HA in the biocomposite.

5.3.3. Mechanical Characteristics of Poly(Vinyl Alcohol)/Hydroxyapatite Scaffold

Compression tests were performed on PVA/HA scaffolds to obtain their mechanical properties. Data of the properties were obtained at the point where the scaffolds reached their maximum compression loading capacity.

Figure 43 shows the sequential compression testing process. All the specimens failed by shearing and cracking at the top, but they did not fragment into smaller pieces.

Figure 43. Sequential process of compression testing: (a) intact specimen, (b) test starts as the compression plate touches the specimen, (c) the specimen is pressed midway, (d) specimen started to fail, (e) failed specimen

Figure 43(a) shows the intact, original specimen. Subsequent pictures display the point when the test commences, which is marked by the compression plate touching the top part

of the specimen (see Figure 43(b)) and the specimen being pressed midway (see Figure 43(c)). Figure 43(d) shows the time when the specimen starts to fail. The failed specimen can be seen in Figure 43(e).

From Figure 43 it is observed that the phenomenon of barrelling was detected as the specimen starts to fail. The specimen illustrates a combination of ductile and brittle characteristics. Figure 43(a)-(c) show the ductility of the specimen, which is caused by the ductile nature of the polymeric matrix. As the plate presses the specimen, the polymer matrix is still able to withhold the stress without breaking. However, as the polymer is sintered to produce the specimen, an attribute of brittleness is introduced to the specimen and the specimen started to fail, as seen in Figure 43(d)-(e). As such failure in shear inside the specimen is expected.

Figure 44 presents the compression stress, strain and modulus results of scaffolds made from pure PVA and PVA/HA biocomposite with 5, 10 and 15 vol.% HA ($n = 15$). In Figure 44(d), a stress-strain curve for 15 vol.% HA samples is given as an illustration of a typical stress-strain curve obtained from the compression test of pure PVA and PVA/HA samples. The compression modulus is taken using the values of compression stress and compression strain from the point where the sample is able to take the maximum load without failure.

As observed from Figure 44, the maximum compressive strength of the scaffolds decreases with the increase of HA concentration. A drop in modulus is also observed in scaffolds with higher concentration of HA, suggesting a decrease in the stiffness of the sample. This suggests that the scaffold's ability to absorb energy without rupturing has deteriorated. The plastic deformation it can undergo has been lessened as well.

Figure 44. Mechanical characteristics scaffolds made of pure PVA, and PVA/HA composites with 5, 10, 15 vol.% HA: (a) Ultimate stress, (b) Strain, (c) Compressive Modulus, (d) Typical stress-strain curve, shown here is for the 15 vol.% HA samples

The reason for the decrease in mechanical properties with the increasing concentration of HA might be due to the fact that HA powder used in the process was not sintered in the SLS process. HA is known to have sintering temperature more than 1200 °C [209], which is much higher than the instantaneous temperature produced in the sintering process in SLS. Therefore in the PVA/HA biocomposite it is the PVA which was sintered and fused together by the SLS process. Hence, the PVA acted as a binder to hold the HA particles, while forming interconnected networks with surrounding PVA particles (see Figure 45(a)).

Figure 45. Model of particle interaction between: (a) PVA-PVA and PVA-HA, (b) PVA-HA, PVA-PVA and HA-HA

When more HA particles are present in a layer, it becomes more difficult for the PVA to bind to the HA particles due to the increase in HA-HA interactions (see Figure 45(b)). HA-HA interactions only perform mechanical interlock among the particles and cannot form any permanent bonding as HA powder are not sintered, thus decreasing the overall binding strength. In the end, there are PVA-PVA, PVA-HA and HA-HA interactions among the existing interactions in the scaffold, but the binding strength is solely contributed by the first two interactions.

The unbonded HA do not have any permanent bonding to the polymer matrix, they introduce voids and discontinuity to the polymeric matrix. As such, the unbonded HA may have acted as stress concentrators inside the sample [39]. Therefore the more volume of HA is added into the scaffold, the more severe the stress concentration. Hence the samples are more likely to fail due to internal crack. The past review has mentioned that there have been divided opinions concerning the addition of a much stiffer and stronger material as compared to the matrix [154]. The mechanical test results of pure PVA and PVA/HA show that the addition of HA, which alone is much stiffer and stronger than PVA, does contribute to a failure mode of the sample. It is a trade-off that should be considered when deciding the necessary amount of HA to be added into the scaffold to increase its osteoinductive nature.

5.4. Summary of Poly(Vinyl Alcohol) / Hydroxyapatite Scaffold Development

Based on the preceding results, it was found that a biocomposite system of PVA/HA has been synthesized and it can be successfully sintered using SLS. The promising sintering of the test specimens indicated the potential of producing PVA scaffolds with SLS.

Further tests showed that the HA in the PVA matrix was not compromised by the mixing or the sintering process. The successful incorporation of HA into the polymer matrix was expected to enhance the bioactivity of the specimens in future *in vitro* experiments, especially with regards to bone-related cells.

PVA scaffold was shown to have compression stress of 3.24 ± 0.72 MPa, compression strain of 0.34 ± 0.12 mm/mm and compression modulus of 10.07 ± 2.35 MPa. The PVA/HA scaffolds were able to give compression stress and compression modulus up to 2.26 ± 0.33 MPa and 8.30 ± 1.1 MPa, respectively, both obtained from the PVA/HA scaffolds with 5 vol.% HA. The best strain obtained from the PVA/HA scaffold was 0.28 ± 0.03 mm/mm, which was given by the scaffolds with both 5 and 10 vol.% HA.

The compressive mechanical properties shown by the scaffolds are in the range of Young's modulus and ultimate stress of trabecular bone without cortical plate in human mandible [187]. Bones for infants with congenital osseous abnormalities may also be potential application, as the requirement of cranium fixation replacement is only for a few weeks at most, coupled with minimal load-bearing function [179]. Therefore craniomaxillofacial skeleton is a prospective application area for the SLS fabricated PVA/HA scaffolds.

Chapter 6

IN VITRO STUDIES OF POLY(VINYL ALCOHOL) BASED TISSUE ENGINEERING SCAFFOLDS

This chapter describes the experiment on the PVA based scaffolds in *in vitro* studies. The initial part of the study is performed by immersing the scaffolds in simulated body fluid (SBF) environment. The later part involves seeding Saos-2 cells onto the scaffolds.

6.1. Bioactivity Analysis of Poly(Vinyl Alcohol) / Hydroxyapatite Biocomposite

Bioactivity analysis was carried out using SBF solution to analyze the influence of HA in the PVA/HA biocomposite. The HA exposure in the scaffolds should stimulate chemical and morphological changes in the samples by mimicking interactions in the body environment. Formation of Calcium (Ca)-rich and Ca-poor amorphous calcium phosphate in HA has been shown to eventually crystallize into bone-like apatite [210].

The SBF solution was prepared by mixing proper proportions of salts in ion-exchanged and distilled water. The salts were sodium chloride (NaCl), sodium hydrogen carbonate (NaHCO₃), potassium chloride (KCl), di-potassium hydrogen phosphate trihydrate (K₂HPO₄ · 3H₂O), magnesium chloride hexahydrate (MgCl₂ · 6H₂O), hydrochloric acid (HCl), calcium chloride (CaCl₂), sodium sulphate (Na₂SO₄) and tris(hydroxymethyl) aminomethane ((CH₂OH)₃CNH₂) [211]. The solution was buffered at pH 7.25.

Ionic concentrations of the SBF were nearly equal to those present in human blood plasma [212, 213], as presented in Table 9.

Table 9. Ion concentration (mM) of SBF and human blood plasma

	Na^{+}	K^{-}	Mg^{2+}	Ca^{2+}	Cl^{-}	HCO_3^{-}	HPO_4^{2-}	SO_4^{2-}
SBF	142.0	5.0	1.5	2.5	148.8	4.2	1.0	0.5
Blood plasma	142.0	5.0	1.5	2.5	103.0	27.0	1.0	0.5

There has been research showing evidence of HA bioactivity when HA is immersed in the SBF environment. A bone-like apatite layer is formed on the surface of ceramic, as a result of calcium and phosphate ions consumption from the body fluid [212, 214].

6.1.1. Simulated Body Fluid Preparation and Experiment Methodology

The SBF was prepared using a protocol detailed by Kokubo [215, 216]. Bottles to be used were washed with 1M-HCl solution, neutral detergent and ion-exchanged and distilled water. To prepare 1 litre of SBF these salt reagents were dissolved in the water: 7.996 g NaCl, 0.35 g NaHCO₃, 0.224 g KCl, 0.228 g K₂HPO₄ · 3H₂O, 0.305 g MgCl₂ · 6H₂O, 0.278 g CaCl₂, 0.071 g Na₂SO₄ and 6.057 g (CH₂OH)₃CNH₂.

The temperature of the solution in the bottle was maintained at 36.5 °C. Solution pH was adjusted by titrating 1M HCl. The SBF has to be stored at 5-10 °C refrigerator when it is not used. Prior to usage, it should be noted that there must not be any precipitation in the solution due to prolonged storage.

Test specimens sintered with the biocomposites were soaked in the SBF for a duration of 15 days and kept in an incubator (Sanyo Incubator CO₂), maintained at 36.8 °C with 3.4% CO₂ injected. The SBF solution was changed every 2-3 days.

Samples that have been immersed in SBF were dried and gold-coated (Bio-Rad SEM coating system) prior to observation by the SEM (JEOL JSM-5600 LV).

6.1.2. Results of Bioactivity Analysis

Figure 46 shows the SEM micrographs of samples after being immersed in SBF. The samples were made of PVA/HA with 5 vol.% HA. Visual observation on 3rd and 5th day showed that the test specimens had swollen. PVA, being a water-soluble polymer, would swell in the presence of fluid. A measurement was carried out using a vernier calliper (Mitutoyo, model no. CD-6" CS). The swelling was noted to be approximately 12% larger in diameter (n = 3). Upon drying, the samples appeared to shrink back to their original size. There was a difference of only 0.2% (n = 3) in diameter between the original and dried samples.

Figure 46. PVA/HA specimens with 5 vol.% HA immersed in SBF: (a) control, (b) at day 3

As shown in Figure 46(b), there is an apparent change on the surface of the specimen, which looks very different from sintered PVA/HA specimens in Figure 46(a). This unique layer was usually located at isolated spots of the samples.

With a high SEM magnification of 1000 times, it is observed that this layer appeared with the characteristic structure of hydroxy carbonate apatite (HCA), which is normally found when HA is soaked in the SBF environment [155, 212]. The presence of HA in Figure 46 is indicated by the rectangular blocks. The surrounding areas of the HA particles in Figure 46(b) have been precipitated into HCA, thus it is likely that these HA are the contributing factors in the HCA precipitation.

The appearance of HCA from HA on the scaffold surface is a sign of calcium phosphate bioactivity. This apatite is a carbonate-containing hydroxyapatite with small crystallites, which is similar to the apatite in the bone in its composition and structure. Bone producing cells preferentially proliferate and differentiate to produce apatite and collagen on this HCA layer. As a result the surrounding bone can come into direct contact with the surface apatite of these ceramics without intervention of fibrous tissue [214].

The appearance of HCA in the scaffold after immersion was further assessed by XRD and FTIR tests. Philips PW 1830 XRD was used to characterize all the samples using step scan with the following parameters: angle $2\theta = 10^\circ$ to 80° , step size = 0.02° , time per step = 1 second, generator tension = 40 kV and generator current = 30 mA.

FTIR (Perkin Elmer FT-IR System Spectrum GX) using the transmission mode of sample preparation was used to check if the PVA material experienced any chemical changes during laser sintering process. Scanning of specimens was performed 4 times, each time from wavenumber 4000 to 400 cm^{-1} . The resolution used was 2 cm^{-1} , with data interval of 0.5 cm^{-1} .

Figure 47 shows the XRD comparison of pure PVA powder, PVA/HA biocomposite with 5 vol. % HA and test specimen after immersion in SBF for 14 days.

Figure 47. XRD comparison of pure PVA, PVA/HA powder with 5 vol.% HA and test specimen after soaking in SBF for 14 days

The presence of PVA in the PVA/HA biocomposite with 5 vol.% HA and test specimen after immersion in SBF for 14 days can be identified by comparing with the respective peaks in pure PVA powder. The peaks occurred at angle 2θ of 11.46°, 19.62°, 22.52° and 40.58°, as indicated by the rectangular blocks. HA's peak in the PVA/HA biocomposite with 5 vol.% HA and test specimen after immersion in SBF for 14 days was identified by referring to JDCPS reference card no. 9-0432 for calcium phosphate hydroxide.

It can be seen that the four highest peaks of HA, as circled, are located at angle 2θ of 25.89° , 31.77° , 32.19° , 32.90° , 46.71° and 50.5° . These HA peaks also occurred at the same or nearby angle 2θ of 14th day SBF sample. Presence of HCA in the SBF sample were identified by HA peaks, as HCA was precipitated from HA in the presence of the SBF. Therefore HCA and HA should have peaks at the same location. The formation of HCA is due to ions exchange between salts originated from the SBF and HA in the PVA matrix [155, 217].

The transformation mechanism of HA into HCA involves three stages [185]. First, crystalline HA transforms into amorphous HA. Upon immersion in the SBF, HA exposes its hydroxyl and phosphate units from its crystal structure. These units give out negative surface charge. The HA surface uses this negative charge to interact specifically with the positive calcium ions in the fluid, consequently forming calcium-rich amorphous calcium phosphate [210].

In the second stage, the amorphous calcium phosphate, which acquires a positive charge, interacts with the negative phosphate ions in the fluid by being dissolved into the surrounding solution, which is the SBF in this case. This results in an over-saturation of the fluid with respect to the apatite [185, 210]. Finally, the apatite grows by consuming the calcium and phosphate ions, and thereby developing the bone mineral-like compositional and structural feature.

It is understood that phosphate, $(\text{PO}_4)^{3-}$, and hydroxyl, $(\text{OH})^-$, compounds in the HA only experience ionic exchange between the SBF environment and the scaffold matrix during the apatite crystals formation [218]. The FTIR spectra, shown in Figure 48, supports this finding. The apatite crystals appeared to contain phosphate and hydroxyl compounds. A similar result was observed for HA that was embedded in the polymer matrix after being immersed in the SBF [139].

Figure 48. FTIR spectra of PVA-HA (10 vol.% HA) specimens after immersion in SBF

The bioactivity analysis has confirmed the HA presence in the PVA matrix. The presence of HA in the polymer matrix has shown its significance by the appearance of crystal-like HCA layer on the scaffold, which was formed when the bioactive HA was under the influence of the SBF. The display of HA bioactivity in providing bone-like apatite, coupled with its natural osteoconductivity [62, 69, 140, 152], have shown that the incorporation of HA in PVA matrix was capable of facilitating bone-related cells to identify themselves better with the scaffolds and in enhancing the cell growth.

6.2. Cell Culture Studies

The cell culture study was conducted to confirm the biocompatibility of PVA-based scaffolds as a living environment for organisms. The experiments in this study were designed to ascertain that the SLS-fabricated scaffolds were safe and would not cause adverse effect to the cells.

6.2.1. Cell Culture Materials, Maintenance and Methodology

Osteoblast-like Saos-2 cells were obtained from European Collection of Cell Culture (ECACC), catalogue no. 89050205. Saos-2 are human primary osteogenic sarcoma cells. This bone tissue was derived from an 11 year-old female Caucasian. Its morphology is epithelial-like [219]. A monolayer of such cell is known to show regular dimensions [220].

Saos-2 cell line was used in preference to harvesting primary culture cells because primary culture cells, in particular osteoblasts, have limited *in vitro* lifetime [221]. Primary culture osteoblasts tend to lose their osteoblastic phenotype in a serial subculture [222]. Therefore obtaining sufficient cells for all cell culture experiments from more than one source may have been necessary, which might have caused a variation in the results.

The Saos-2 cell line has been reported to release bone inducing agent that stimulates ossification when implanted subcutaneously in nude mice. The secreted bone inducing agent consists of a mixture of vesicles and they resemble the matrix vesicles of dentin, cartilage and bone [223, 224]. Unlike other lines of human sarcoma cells, Saos-2 reflects an advanced state of differentiation by showing non-tumorigenicity characteristics [223].

Typical growth cycle of cells can be divided into three different phases, namely lag phase, log phase and plateau phase [220, 225]. A typical cell growth curve is illustrated in Figure 49.

Figure 49. Typical cell growth curve [225] (Source: Freshney. Maintenance of Primary and Early Passage Culture. In Atala, *et al.* Methods of Tissue Engineering, 2002, p. 37-53)

The first phase is the lag phase, when the cells start to attach onto a substrate and spread out. There is usually a lag in cell growth and no apparent increase in cell concentration before significant growth begins [220, 226].

The second phase is the log phase, when the cells are growing and population doublings occur. This is the time when the cells are in their most reproducible forms [220]. Cells normally double their density every 18-36 hour until they become confluent [226]. Confluence in a monolayer culture is signified by cells covering the entire available substrate. The time taken to conclude the log phase depends on the cell seeding density onto the substrate.

The third phase is the plateau phase, which occurs shortly after confluence is reached. This is the stage when growth rate ceases or is greatly reduced [220]. Cell growth at this stage is limited by various circumstances: nutrients in the culture media have been depleted to a level that cannot sustain further cell growth, accumulation of cell by-products have

reached the level that is inhibitory to cell growth and cells have passed their confluence state [226].

Cell culture study in this research was performed following a systematic procedure illustrated in Figure 50. The detailed methodology will be described in the following sections.

Figure 50. Process flow in cell culture study

6.2.1.1. Cell Culture Maintenance

The culture medium is an essential part of cell culture as this chemically complex fluid is responsible for supporting cell growth, division and expression of unique characteristics [220, 227]. The media used was α -Minimum Essential Media (MEM) (Gibco, Invitrogen Corporation, catalogue no. 12571-063). MEM contains amino acids, vitamins and inorganic salts [228]. The MEM used for monolayer culture had phenol red as pH indicator of the medium and accounted for the colour of the culture media. Normal pH of cells and their environment in most mammalian species is 7.2-7.4 [220]. At a lower pH of 7.0 the phenol red becomes orange and at pH 6.5, yellow. A medium becomes exhausted and acidic, indicated by change in colour into yellowish, when cells are very densely populated, indicating a need for subculture or medium change [220]. The media for Saos-2 culture was changed every 3-4 days.

Serum is a complex mix of albumins, growth factors and growth inhibitor. Its function is to increase the buffering capacity of cultures, protect cells against mechanical damage and bind and neutralize toxins [229]. The serum used was fetal bovine serum (FBS) (Gibco, Invitrogen Corporation, catalogue no. 26140-087).

Adding a supplement of antibiotics, such as penicillin and streptomycin, in cell culture media has become common since 1950s. Biological fluids are particularly vulnerable to bacteria contamination even in a controlled environment. The antibiotics used (Gibco, Invitrogen Corporation, catalogue no. 15140-148) contains 10,000 units of penicillin (base) and 10,000 μ g of streptomycin (base)/ml utilising penicillin G (sodium salt) and streptomycin sulphate in 0.85% saline. Penicillin G is used for protection against Gram-positive bacteria and streptomycin protects the culture from Gram-negative and Gram-positive bacteria [226].

Saos-2 cells were grown as monolayer cultures in a concoction of α -MEM supplemented by 10% (volume/volume) FBS and 1% (volume/volume) penicillin-streptomycin. Cultured cells were kept in an incubator (JISICO J-2000), which was set at 36.5 °C and 4.0% CO₂. All cell culture work was carried out in a Gelman Class II Biosafety cabinet.

Culture cells are ideally subcultured at the end of log phase before they enter plateau phase (see Figure 49). It is harder to disaggregate cells in plateau phase and cells subcultured in such condition are likely to experience longer time in lag phase [225]. Saos-2 cells were subcultured every 7 days in a proportion of 1:5.

6.2.1.2. Cell Counting

The concentration of a cell suspension was quantified by using a hemacytometer slide. Of the many types available, the Improved Neubauer model is the most commonly employed. The Improved Neubauer hemacytometer used was supplied by Marienfeld, Germany. The procedure to use a hemacytometer is illustrated in Figure 51.

Trypan blue (Sigma, catalogue no. T8154) was used as an exclusion dye for accessing the health and viability of cells in conjunction with a counting chamber [220, 230]. Healthy cells exclude the dye and are shown unstained and clear with a refractile ring around them (see Figure 51(h)). Dying cells will take up the dye and are seen to have a dark colour without a refractile ring. Trypan blue is normally added to the cell suspension to make a 4-time dilution.

The counting chamber depth is 0.1 mm for an Improved Neubauer slide, therefore the volume of each 1 mm² chamber is 0.1 mm³ or 1x10⁻⁴ ml. Cells are normally counted from five 0.1 mm³ chambers, indicated by number 1 to 5 in Figure 51(f), obtaining a total volume of 5 x 10⁻⁴ ml cell suspended. With a dilution factor of 4 times, cell density of the suspension can be calculated as follows:

$$\text{Cell density (cells / ml)} = \frac{\text{Total live cells}}{5} \times 4 \times 10^{-4} \quad (17)$$

To calculate cell viability, the formula used is as follows:

$$\text{Cell viability (\%)} = \frac{\text{Live cells}}{\text{Total cells}} \times 100\% \quad (18)$$

Figure 51. Using a Hemacytometer slide [231]: (a) A hemacytometer slide and coverslip before use, (b) pressing cover slip onto slide, (c) adding a cell suspension to the assembled slide, (d) longitudinal section of the slide, showing the position of cell sample in the 0.1-mm-deep chamber, (e) viewing slide on microscope, (f) magnified view of the grid total area, (g) Low-power (10x objective) photo showing the 25 smaller 200x200-μm squares of the slide, (h) High-power (40x objective) photo of one of the smaller 200-μm square (Source: Freshney. *Culture of Animal Cells: A Manual of Basic Technique*, 2005)

6.2.1.3. Detection against Contamination

Bacteria and fungi in mammalian cell cultures can be observed when there is a sudden drop of pH and indicated by a murkiness of the medium. A culture with phenol red added media is likely to be contaminated if the culture becomes murky and changes from red to yellow overnight [226].

Mycoplasmas are Gram-negative bacteria, with size approximately 0.3-0.5 μm and the capability to grow within the cell cytoplasm. They are slow growing and thus unlikely to overgrow the cell culture, but they may affect the cellular growth rate, morphology, viability and metabolism [226]. This type of contamination is very difficult to detect in a cell culture, especially just with naked eyes.

Mycoplasma detection was performed using DNA fluorochrome staining. With this method, a cell monolayer that is 50-80% confluent was fixed and stained with a DNA specific dye and examined using a fluorescent microscope. For the detection Saos-2 cells were stained using Hoechst stain solution 0.5 $\mu\text{g/ml}$ (Sigma, catalogue no. H6024) diluted 10 times in Hanks' Balanced Salt Solution (Sigma, catalogue no. H5899).

A fluorescence microscope (Olympus BX 51) was used to examine the stained culture. A negative culture shows only nuclear staining, while a positive culture shows particulate of filamentous fluorescence around the cell nuclei. A heavy infection of mycoplasma normally stains the inter-cellular spaces. Mycoplasma positive and negative control slides were obtained from Sigma (catalogue no. M1414).

6.2.1.4. Measurement of Cell Proliferation

An assay using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide] was used to study the cell proliferation and approximate the cell quantity on the scaffolds.

MTT assay involves a reduction reaction which reduces the MTT reagent to formazan crystal product of dark purple colour when incubated with living cells. This is a result of mitochondrial dehydrogenase of the cells. The MTT is initially a colourless compound. The purple crystals are solubilised by lysing buffer and the intensity is measured

colometrically to give a quantitative measure of cell viability. The absorbance of formazan indirectly reflects cell metabolism level [232, 233].

MTT (Sigma, catalogue no. M2128) with a concentration of 5 mg/ml in phosphate-buffered saline (PBS), pH 7.4 (Sigma, catalogue no. P3813), was used in the measurement. The MTT solution was added onto the culture well in an amount equal to 10% of the culture volume. The culture was then incubated for 3-4 hours. After the incubation period, lysing buffer was added in an amount equal to the original culture volume to dissolve the resulting MTT formazan crystals. The colour intensity was measured using an ELISA (Enzyme Linked Immunoabsorbance Assay)-reader (Bio-Rad, Benchmark Plus) at a wavelength of 570 nm. The media used for MTT assay test was without phenol red (Gibco, Invitrogen Corporation, catalogue no. 41061-029), so as to minimise the colour intensity interference during measurement.

6.2.2. Saos-2 Cell Characterizations

Cell viability of Saos-2 was calculated using formulae (18). Average viability of cultured Saos-2 at confluence was 86%. Average cell density was recorded to be 1.84×10^6 cells/ml. The cultured cell density was considered sufficient for the seeding experiment onto the biopolymer scaffolds [234].

Quality control test of Saos-2 cell condition showed negative results for mycoplasma contamination. Figure 52 shows the light and fluorescence microscope images of the test.

Figure 52. Microscope images of Saos-2 mycoplasma test result, in bright field and fluorescence: (a)-(b) positive control, (c)-(d) negative control, (e)-(f) Saos-2 cells

By comparing the Saos-2 slides (Figure 52(e) and (f)) to the control slides it was found that the Saos-2 cells resembled the images of the negative control (Figure 52(d) and (e)), with only nuclear staining shown by the dark-blue fluorescent light. There were no stains in the inter-cellular spaces, as can be seen in heavily infected cells of the positive control slides (Figure 52(a) and (b)). The cells were deemed healthy and free from contamination and hence they can be used for seeding.

6.2.3. Cell Observation Methodology

Observations were carried out to study the cell behaviour on the scaffolds, including cell morphology on the scaffolds and verification that the cells were present within the 3D structures of the scaffolds.

6.2.3.1. Observation of Cell Existence inside 3Dimensional Scaffolds

DAPI (4',6-diamidino-2-phenylindole) is a blue stain and is used to view organisms' nucleus under fluorescent light [230, 235]. The stain was used to determine if seeded cells could migrate into the depth of the scaffolds and not only just on their surfaces. This is important as cell migration into the inner part of the scaffold is an issue to ensure that nutrients and oxygen are circulated thoroughly within the scaffolds [35, 236]. This is because when cells only attach on the scaffold surface, they consume the most nutrients and oxygen. Hence it limits the amount available for pioneering cells that are migrating deep into the scaffold. Eventually further cellular migration is halted due to the lack of oxygen and nutrient supply [35]. As the cells migrate into the inner part of the scaffold it is easier to form one target organ and to have nutrients circulating in the scaffold.

SLS-fabricated 3D scaffolds are not transparent, therefore fluorescent light from the microscope is unable to penetrate through the scaffold. To obtain a clear observation of the inside, the scaffolds have to be sliced.

To facilitate slicing, the scaffolds were embedded in PBS solution consisting of 5% (weight/volume) gelatine (type A from porcine skin, Sigma, catalogue no. G-1890) and 5% (weight/volume) sucrose (Sigma, catalogue no. S5391). A cryostat (Leica, model CM3050S) was used to cut the scaffolds into slices of 7-10 μm thickness. The specimen temperature was set to -20 $^{\circ}\text{C}$ while the cryo chamber temperature was set to -15 $^{\circ}\text{C}$.

DAPI stock (Aldrich, catalogue no. D9542), diluted in PBS in concentration of 300 nM, was applied onto the sliced specimens. The specimens were then incubated for 3 minutes and washed with PBS to remove all the remaining DAPI solution. The fluorescence microscope (Olympus BX 51) was set for observation at excitation and emission wavelengths of 340 and 488 nm, respectively.

6.2.3.2. Observation of Seeded Cell Morphology

SEM was used to observe seeded Saos-2 cell morphology on the scaffolds. The cells on the scaffolds were fixed in 100% methanol (Merck, catalogue no. 106009) for 30 minutes at 4 °C environments. After the specimens were rinsed with PBS, a series of ethanol dehydrations were carried out to dry them [237]. The specimens were dehydrated in 30%, 50%, 70%, 80%, 90% and 100% ethanol, each for a duration of 15 minutes. The final drying process was carried out using a critical point dryer (Bal-Tech, model CPD030).

6.2.4. Preliminary Cell Seeding on Poly(Vinyl Alcohol) Scaffolds

There were 2 types of PVA scaffolds used for the preliminary cell seeding: SLS-fabricated PVA scaffolds and casted PVA thin film. This pilot experiment was carried out to ascertain if the cells would proliferate better in 3D structures, represented by the SLS-fabricated scaffolds rather than on the surface, represented by the thin films.

Prior to seeding the scaffolds were sterilized in 70% ethanol for 3 hours. To ensure the ethanol was thoroughly removed, the scaffolds were washed and immersed in PBS for 3 hours. Afterwards the scaffolds were conditioned by overnight immersion in culture media one day before seeding. The conditioning was to help cells recognize the substrate as part of their imminent living environment.

The SLS-fabricated scaffolds were built with laser power 13 W and scan speed 2032 mm/s. The PVA thin films were prepared by dissolving PVA powder (99% + hydrolyzed, average molecular weight of 89,000-98,000, from Aldrich) in deionised water. Afterwards the dissolved PVA was spread evenly onto a flat acrylic board. It was left for a few hours under room temperature to dry it and upon drying the film was cut into the required dimension. The PVA concentration in the thin film was 36% (weight/volume). All the scaffolds had a height of 1.5 mm and a diameter of 13 mm. The dimensions were designed so that they could fit snugly in a 24-well tissue culture plate (TPP, catalogue no. 92024).

Cells with density of 1×10^6 cells/ml were seeded onto each of the scaffolds and kept for 8 days. Media for the cells were changed every 2-3 days. Three different controls were used.

Control 1 was a blank control of media without cells. Control 2 was a well-plate with cells seeded on the polystyrene (PS) base of the well. Control 3 was a blank scaffold without cells. The number of repetitions for both scaffolds and controls was 5 ($n = 5$).

Figure 53 shows the MTT results of the seeded scaffolds and controls. In areas where the cells were populated, a dark colour MTT stain on the substrates could be seen.

Figure 53. MTT result of PVA scaffolds (SLS-fabricated and thin films). Control 1: blank control without cells. Control 2: cells seeded on PS (well-plate). Control 3: blank scaffold without cells.

Sample: scaffold seeded after 2 and 8 days, respectively.

The analysis showed that the cells could survive on the SLS-fabricated PVA scaffolds for the entire seeding period. The dark colour stain on the 3D scaffolds was a distinct contrast compared to the unstained control. However, it was observed that there were no signs of cells attaching and proliferating on the thin film surface. The colour of the seeded films was very similar to that of control 3 of the thin film. The result was further verified from the quantitative absorbance reading, as shown in Figure 54.

Figure 54. MTT reading of Saos-2 cells seeded on various substrates

As seen in Figure 54, the dark purple colour intensity reading in the seeded films (pink colour) is very similar to those of the thin film control (green colour). This indicates that there was limited cell attachment and proliferation on the thin films. On the contrary, encouraging signs of cell growth were seen on the 3D SLS-fabricated scaffolds, with its relative intensity absorbance surpassing that of the intensity reading from the PS well. The result indicated that the likelihood of Saos-2 cells to attach, grow and proliferate on SLS-fabricated scaffold was higher than on the thin film.

SEM observation of seeded SLS-fabricated scaffolds, as seen in Figure 55, affirmed the MTT results. The cells could be easily spotted on the scaffolds. Over the culture period the cells could attach and spread themselves on the scaffold, as shown in Figure 55(a) and (b), over a localised surface area of the scaffold. This showed that Saos-2 could adapt itself well onto the PVA matrix.

The osteoblast-like cells had established their tentacle-like enlargement, shown by the arrows and these are known as filipodia [238], to attach themselves onto the substrate, as seen in Figure 55(c). The 3D phenotype cell morphology is shown in Figure 55(d).

Figure 55. Saos-2 cells on SLS-fabricated 3D scaffolds: (a) day 2, (b) day 5, (c) cell attachment onto the scaffold, (d) 3D phenotype morphology of Saos-2 cell

It was noted from that the cells were found to envelope predominantly on the areas of partially sintered particles. The cells in Figure 55(a) and (b) are seen to attach and spread on such surfaces.

Cell clumps were spotted at some areas of the scaffolds, such as shown in Figure 56(a) and indicated by the arrows. They clustered and attached on the partially sintered areas of the scaffold. The cell clump morphology is shown in Figure 56(b) and it shows slightly different phenotype from when the cell has spread out as shown in Figure 55(d).

The reluctance of the cells to attach on fully sintered areas is further shown in Figure 56(c) by the gap between the cell clusters and the fully sintered surface. From Figure 56(d), it is clearly seen that fully sintered particles gave a smoother surface compared to partially sintered particles. The cell adherence preference suggested that there the Saos-2 cells had shown preference for surfaces with certain surface roughness.

Figure 56. Phenomena of cells in SLS-fabricated 3D scaffolds: (a) cell balls on partially sintered particles, (b) 3D phenotype of cell balls, (c) gap between cells and fully sintered surface, (d) cell balls attaching on partially sintered surface

Attachment and proliferation of osteoblast-like cells is reported to increase as a function of surface roughness and is greatest on the rougher surface with more irregular topographies [239]. Surface roughness is also found to promote osteogenic differentiation [240, 241]. The difference in surface roughness of fully sintered and partially sintered area could possibly be the reason that explains the preference of Saos-2 attachment and proliferation.

Qualitative observation of PVA thin film under SEM showed that the film possessed similar, if not less, surface roughness compared to the fully sintered area in SLS-fabricated scaffold. It was very rare to locate any cells on the thin film. A few cells were found attaching on the curves or dent parts of the film, as seen in Figure 57. These curvatures gave a less smooth area of the film and therefore seemed to have attracted the cells to attach themselves there.

Figure 57. Secluded cells attaching on curves or dent parts of PVA thin film, shown with magnification: (a) 100 times, (b) 250 times

Cell colonisation at the scaffold periphery has been addressed in previous reports [35]. Cells were unable to migrate deep into the scaffolds because of the lack of nutrients and oxygen and insufficient waste product removal. It also generates another problem as they are acting as an effective barrier to the diffusion of nutrients and oxygen into the interior of the scaffold [236].

Figure 58 shows the inner level of the SLS-fabricated scaffolds, seen from its cross section. The arrow points to a cluster of cells attaching on some part of the scaffold network.

Figure 58. Cells at inner level of the SLS-fabricated scaffolds, seen from the cross sectional area

The existence of cells at the inner levels of SLS-fabricated scaffold was further ascertained with observation using DAPI stain, as shown in Figure 59.

Figure 59. Fluorescence microscope images: (a)-(b) scaffold control without any cells, (c)-(d) seeded scaffold at lower levels, showing cell nuclei

Figure 59(c) and (d) show fluorescence images of the SLS-fabricated scaffold at the lower levels. DAPI staining of thin scaffold layer shows blue colour to indicate the cells' nuclei and green colour background to indicate the surrounding PVA porous network. It can be seen from Figure 59(c) that the cell balls clusters appear to be distributed over the entire area of the scaffold.

The occurrence of the cells in the inner layers of the SLS-fabricated scaffolds showed the structural strength of the scaffolds. The interconnected porosity within the scaffolds has favoured seeded cells to migrate into the deeper part of the 3D structure and be distributed inside the scaffold. This shows the potential of SLS-fabricated scaffolds to fabricate interconnected scaffold that is able to overcome the cellular barrier problem.

6.2.5. Cell Seeding on Poly(Vinyl Alcohol) / Hydroxyapatite Scaffolds

PVA/HA (with 5 vol.% HA) and pure PVA scaffolds were used as substrates for cell seeding in this experiment. The scaffolds were built with laser power 13 W and scan speed 2032 mm/s. The scaffolds were identical in shape and dimensions, having a height of 1.5 mm and a diameter of 13 mm.

Saos-2 cells with density of 1×10^6 cells/ml were seeded onto each of the scaffolds and kept for a duration of 8 days. Media for the cells were changed every 2-3 days. Three different controls were used. Control 1 was a blank control of media without cells. Control 2 was a well-plate with cells seeded on the polystyrene (PS) base of the well. Control 3 was a blank scaffold without cells. The number of repetitions for both scaffolds and controls was 4 ($n = 4$).

MTT assay tests showed that all the PVA/HA scaffolds were able to act as substrates for the Saos-2 cells to survive for the entire culture period. The PVA/HA scaffolds, as seen in Figure 60, were stained dark purple indicating live cells were occupying the 3D structures.

Figure 60. MTT results of PVA and PVA/HA scaffolds. Control 1: blank control without cells. Control 2: cells seeded on PS (well-plate). Control 3: blank scaffold without cells. Sample: scaffolds seeded after 2 and 8 days, respectively.

A calibration curve was plotted to approximate the number of cells represented by the MTT absorbance intensity (Figure 61).

Figure 61. MTT calibration curve

The relationship between the relative absorbance intensity and the number of cells is given as follows,

$$x = \frac{y - 1.4672}{0.00004} \quad (19)$$

x = the number of cells

y = relative absorbance intensity

Equation (19) was used to determine the number of cells on each scaffolds based on the reading of the relative absorbance intensity. The approximate number of cells on the scaffolds over the culture period of 8 days is summarized in Figure 62.

Figure 62. Approximate number of cells on scaffolds over 8 days of culture (The sign * denotes significant difference, $p < 0.05$)

From the graph shown in Figure 62 it can be seen that the amount of cells cultured in monolayer on the PS well constantly drops during the culture period. In a period of less than 4 days, the cells experienced a declining phase, which indicated the possibility of cell death. The measured viable cell concentration decreases as the cells lyse. This is possibly due to cell apoptosis initiated by the depletion of nutrients and lack of growing space as the cells in the well have gone passed their confluence stage in the monolayer culture. Accumulation of metabolic by-product may have reached a level that inhibits cell growth. These conditions will trigger the cell concentration to decline rapidly [226]. This finding is similar to previous results of Saos-2 experiencing a decline in number over the period of seven days when cultured on PS well [234].

The cells on the multilayer culture, however, have managed to proliferate and quantitatively increase for at least 4 days. The network porosity of the scaffolds has allowed nutrients and oxygen to be better distributed than in a monolayer culture. It was observed that both types showed a reduced number of cells after the same period of time. As the scaffolds were designed with uniform size, the decline in the amount of cell after 4 days was likely to be caused by the size of the scaffolds that were unable to hold the cell

growth any longer. As such the cells found themselves constrained for further growth after this period of time.

The number of cells in the PVA/HA scaffolds was monitored and found to be constantly more than those on the PVA scaffolds. HA, being a bioactive ceramic, induces the formation of hydroxy carbonate apatite crystals on HA biomaterial surface, which has been established in previous experiment (see Chapter 6.1). The carbonated HA mediates the effects on osteoblast-like cell activities, including initial attachment and subsequent proliferation [140, 242], thus enhancing the cell growth.

SEM micrographs were used to view the adherence of the cells on PVA/HA scaffolds. The image of PVA/HA control scaffold is shown in Figure 63. From the images, it is clearly seen that the HA particles, indicated by the arrows, were well exposed in the sintered PVA matrix.

Figure 63. PVA-HA control scaffold

Saos-2 cells were able to adhere and spread well on the PVA/HA scaffolds. It can be seen from Figure 64(a) and (b) that the cell spread in these HA-incorporated scaffolds was more encouraging than on the ones without HA (see Figure 56). Many of the cells were able to divide themselves and proliferate on the areas of fully sintered PVA matrix, unlike the phenomena observed on pure PVA scaffolds.

Figure 64. Cell adhesion on PVA/HA scaffolds: (a)-(b) cells spread on the scaffold, (c) cells adhere on surface where HA particles are incorporated, (d) cells follow the contour of scaffold

The ability of the cells to attach and proliferate onto the sintered PVA matrix was likely enhanced by the encapsulation of HA in the PVA matrix. These osteoblast-like cells were able to recognize the bioactivity that was exhibited by the HA materials [140, 242]. Figure 64(c) visibly illustrates that the cells spread on the areas where HA particles are encapsulated in the PVA sintered network.

The manner on which the Saos-2 cells proliferated on the PVA/HA scaffolds was observed to be different from that on pure PVA scaffolds. Figure 64(d) shows that the proliferation of the osteoblast-like cells follow the contour of the PVA/HA scaffolds. This indicates that the distribution of HA inside the scaffolds was relatively even throughout the PVA matrix, hence giving the cells “direction” to follow the matrix contour.

The PVA/HA scaffolds were able to provide interconnected network to allow cell migration inside the scaffolds. Figure 65 shows an image of cells that are in the process of

penetrating from the topmost surface of the scaffold into the inner parts of the scaffold. The cells appeared at both the top surface as well as the cross section (inner part) of the scaffold.

Figure 65. Cells that are penetrating into the inner part of PVA/HA scaffold

Other evidence that the cells were found in the inner parts of the scaffolds is shown in Figure 66. As the seeded scaffolds were sliced into 10 µm-thick specimens and stained with DAPI, the fluorescent images showed that cell nuclei were spotted randomly throughout the sliced specimens. The existence of the cells in the inner parts of the PVA/HA scaffolds showed that the porous network generated from the SLS process was able to provide sufficient passages for the cells to migrate.

Figure 66. Fluorescent images of scaffolds with DAPI stain showing cell nuclei, with magnification:
(a) 4 times, (b) 10 times

6.3. Summary of In Vitro Studies on Poly(Vinyl Alcohol)-Based Scaffolds

The *in vitro* studies of both the SBF and cell culture have shown favourable results. The incorporation of HA in the scaffolds has enhanced the initial attachment of cells and subsequently the proliferation and distribution of the cells in the scaffold. The HA has also affected the manner and direction in which the cells migrated. These positive results were due to the bioactive nature of HA.

SLS-fabricated scaffolds have been shown to be able to provide a favourable living environment for the osteoblast-like cells. The use of 3D scaffolds to host a multilayer culture has improved the growth and proliferation of Saos-2 cells in a prolonged period of time in these 3D scaffolds compared to a monolayer culture. The porous network of the PVA matrix has also been shown to have enabled the flow of oxygen and nutrients to be thoroughly distributed inside the scaffolds. Hence the cell growth was supported until the period where the scaffold size could no longer accommodate the quantity of cells.

The seeded cells have been found at the inner parts of the scaffolds and not only just accumulating at the top-most layer of the scaffolds. This showed that the porous network obtained from the SLS fabrication was sufficient to allow cell migration. As such, the fabrication method was capable in providing good scaffold that could overcome effective barrier problem. This effective barrier has been reported to block the diffusion of nutrients and oxygen into the interior of the scaffold [35].

These findings ascertained the feasibility of PVA to be processed on the SLS without resulting in any adverse effects for mammalian cell culture. This study served as a foundation that sintered synthetic polymers, such as PVA, were able to support cell growth.

Chapter 7

CONCLUSIONS AND FUTURE WORK

This chapter summarises all the work carried out throughout the Ph.D. project. Substantial future works would also be presented. Lastly, published work based on this research is listed.

7.1. Conclusions

The theoretical study on heat transfer phenomena in selective laser sintering (SLS) is first summed up. Secondly, development of poly(vinyl alcohol) (PVA) and hydroxyapatite (HA) biocomposite is presented. Thirdly, the feasibility of scaffold fabrication using SLS is given. Lastly, the biocompatibility of SLS fabricated tissue engineering scaffolds *in vitro* is highlighted.

7.1.1. Theoretical Study on Heat Transfer Phenomena in Selective Laser Sintering

A theoretical study on heat transfer phenomena in SLS was carried out to understand the sintering process. The fundamental law of *Conservation of Energy for a Control Volume* was applied to describe the heat transfer phenomena and derive the corresponding governing equations. Sintering in SLS environment was modelled as a control volume with the periphery of the powder bed as the control surface.

The derived theoretical heat transfer equation established that both laser beam and material properties contributed to the energy intensity obtained by the powder during sintering process. The influential material properties were thermal conductivity, thermal diffusivity, specific heat, powder reflectivity and effective absorption coefficient. The predominant laser beam properties were laser power and scan speed. These parameters were user-controllable and could be used to reduce the number of sintering trials to obtain favourable sintering windows for processing biomaterials in SLS.

A laser beam temperature distribution simulations and measurements, followed by verification of sintered biopolymer powder in SLS, were carried out. The laser power was varied while the scan speed was kept constant throughout this study. Both the simulation and measurement results showed that there was a significant difference in maximum temperature as the laser power was varied. The SLS processed biopolymer affirmed these results. It showed that with higher energy density obtained by the polymer powder, the sintering resulted in a greater neck width but a smaller pore size. This study has provided the users with the flexibility of controlling the machine parameters to obtain the desired degree of sintering.

7.1.2. Development of Poly(Vinyl Alcohol) / Hydroxyapatite Biocomposite

Two different methods were investigated to develop a biocomposite comprising of PVA and HA biomaterials for TE scaffold application, namely spray-drying and mechanical mixing. The results for these methods varied from inadequate sintering effects to good sintering identified by the formation of necking. The process was capable of creating an interconnected network in a scaffold. Favourable sintering windows for each composition of PVA/HA biocomposite have been obtained.

The most prominent sintering was obtained from PVA and HA biocomposite obtained by mechanical mixing of as-received PVA and HA powders, regardless of the PVA and HA proportion in the biocomposite. The presence of PVA and HA particles in the sintering network could be clearly distinguished. The exposure the HA particles could be easily identified. This is important to encourage cell proliferation further *in vitro* studies [65].

A separate study showed that the mechanical mixing method employed in this research has yielded a homogenous biocomposite with relatively high repeatability. Hence, the PVA/HA biocomposite was prepared by the mechanical mixing method to fabricate subsequent samples for further characterization.

Characterization of PVA/HA scaffolds showed good results. The chemical composition of PVA as the scaffold matrix was not affected during the sintering process. Sintering process also did not affect both the composition and the quantity of PVA and HA in the biocomposite. The PVA/HA scaffolds gave compression stress and compression modulus

up to 2.26 ± 0.33 MPa and 8.30 ± 1.1 MPa, respectively, both were obtained from PVA/HA scaffolds with 5 vol.% HA. The compressive mechanical properties shown by the scaffolds were in the compressive strength range of trabecular bones without cortical plate in human mandible. Therefore applications of PVA/HA scaffolds targeting cells at the craniomaxillofacial areas are theoretically feasible.

7.1.3. *In Vitro* Biocompatibility of Poly(Vinyl Alcohol) / Hydroxyapatite Scaffolds Fabricated by Selective Laser Sintering

In vitro studies were carried out by immersing the PVA/HA scaffold in simulated body fluid (SBF) and seeding osteoblast-like Saos-2 cells. Satisfactory results were obtained from these studies. The SBF experiments established that incorporating HA in the polymer-based scaffold was significant. This was affirmed by the appearance of bone-like hydroxycarbonate apatite layer on the scaffold, which was precipitated when bioactive HA was exposed in the SBF environment.

The use of SLS fabricated 3D scaffolds to host a multilayer culture has improved the growth and proliferation of Saos-2 cells in a prolonged period of time compared to a monolayer culture. The porous interconnected network in the SLS fabricated scaffolds has enabled oxygen and nutrient flow to be thoroughly distributed in the scaffolds. This was important to support cell proliferation.

The seeded cells migrated into the inner part of the scaffolds and did not only just accumulate at the surface of the scaffolds. This showed that the interconnected porous network achieved by the SLS process was sufficient to allow cell migration. It also displayed that the SLS fabricated scaffolds were capable of providing an effective solution to overcome the cell barrier problem that was previously reported to block oxygen and nutrient diffusion into the interior of a scaffold [35].

Saos-2 cells were found to attach easily and proliferate on the PVA/HA scaffolds rather than the pure PVA ones. This showed that the incorporation of HA in the scaffolds has enhanced the initial cell attachment and subsequent proliferation and distribution of cells in the scaffold. The HA in the PVA/HA scaffold was also found to affect the manner and direction of cell movement.

These favourable findings ascertained the feasibility of PVA/HA biocomposite to be processed in SLS without generating any adverse effect for mammalian cell culture. This study contributed to the scientific knowledge that sintered synthetic biomaterials, such as PVA, were able to provide a living environment for cells and support the cell growth.

7.2. Future Research Directions

The following future research directions are recommended:

7.2.1. Heat Transfer Modelling of Selective Laser Sintering

Current theoretical study of heat transfer phenomena in SLS with respect to biomaterials has identified the significant material and laser beam properties. The significant laser beam properties were used to reduce subsequent sintering trials to obtain the favourable sintering windows. A possible improvement includes the elimination of sintering trials. This can be carried out by relating the energy density obtained by the biomaterials during sintering with the expected pore size formed from the sintering.

Secondly, heat transfer modelling can be carried out for processing biocomposite in SLS. The current study was limited to the heat transfer phenomena in one polymeric material during sintering. Further study could address biocomposite comprising of different polymers or polymer-ceramic composite.

The current model has not discussed the aspect of heat effect obtained between adjacent laser trajectories. In the current model and experimental data the hatch spacing, which is the overlap distance between the lines traced by the laser, is not included to reduce the complexity of the model. The hatch spacing has been kept as a constant according to the default material used. The planning of different hatch spacing values and distribution could be highlighted for further optimization of the SLS process control.

7.2.2. *In Vitro* and *In Vivo* Biocompatibility Tests for Scaffold Fabricated by Selective Laser Sintering

In vitro experiments that have been carried out in this project involved cell culture in a static environment. Further *in vitro* experiments can be implemented in a dynamic environment, such as using a bioreactor. The behaviour of seeded cells on scaffolds is expected to be different in static and dynamic environments. This would lead to development work of suitable bioreactors to be used for PVA/HA scaffolds. A requirement includes the bioreactor's capacity to ensure controlled nutrient and oxygen flow in the scaffolds, without damaging the scaffolds.

As PVA/HA scaffolds have been shown to have the capability of providing satisfying living environment for Saos-2 cells *in vitro*, further study can be focused *in vitro* test using primary cell lines, followed by *in vivo* tests using animal models. Possible targeted area is bone with similar mechanical strength given by PVA/HA scaffolds.

Histological-type work can be carried out to investigate the path taken by the cells *in vitro* to go into the depth of the 3D scaffolds. In *in vivo* tests, histological-type works would be useful to study the cell and tissue structures that would attach onto the scaffold.

7.3. List of Publications

This project has won the Gold Prize – Engineering Innovation Award in the Project Competition of the College of Engineering Technology Exhibition in 2005. In addition, a number of publications have been produced based on this research work. The publications include 5 international journal papers (3 published and 2 accepted for publication) and 5 international conference papers.

Award:

- F.E. Wiria, Z.Y. Quah, C.K. Chua, K.F. Leong, M. Chandrasekaran, M.W. Lee, “Theoretical Modeling of Selective Laser Sintering Heat transfer Phenomena for Biomaterials”. Presented at College of Engineering Technology Exhibition, 9-13 March 2005, Nanyang Technological University, Singapore. *This research work was awarded the Gold Prize – Engineering Innovation.*

International Refereed Journal Papers:

1. F.E. Wiria, C.K. Chua, K.F. Leong, Z.Y. Quah, M. Chandrasekaran, M.W. Lee. “Improved Biocomposite Development of Poly(Vinyl Alcohol)/Hydroxyapatite for Tissue Engineering Scaffolds Fabrication using Selective Laser Sintering”. *Journal of Materials Science: Materials in Medicine*. Published Online (2007). – Impact factor: 1.562.
2. R.L. Simpson, F.E. Wiria, A.A. Amis, C.K. Chua, K.F. Leong, U.N. Hansen, M. Chandrasekaran, M.W. Lee. “Development of a 95/5 Poly(L-Lactide-co-Glycolide) / Hydroxyapatite & β -Tricalcium Phosphate Scaffold as Bone Replacement Material via Selective Laser Sintering”. *Journal of Biomedical Materials Research – Part B: Applied Biomaterials*. Published Online (2007). – Impact factor: 1.778.
3. K.F. Leong, F.E. Wiria, C.K. Chua, S.H. Li. “Characterization of Poly- ϵ -Caprolactone Polymeric Drug Delivery Device Built by Selective Laser Sintering”. *Bio-Medical Materials and Engineering* 17, 147-157 (2007). Impact factor: 0.922.
4. F.E. Wiria, K.F. Leong, C.K. Chua, Y. Liu. “Poly- ϵ -Caprolactone/Hydroxyapatite for Tissue Engineering Scaffolds via Selective Laser Sintering”. *Acta Biomaterialia* 3, 1-12 (2007). – Impact factor: 2.132.
5. K.H. Tan, C.K. Chua, K.F. Leong, C.M. Cheah, W.S. Gui, W.S. Tan, F.E. Wiria. “Selective Laser Sintering of Biocompatible Polymers for Applications in Tissue

Engineering”. *Bio-Medical Materials and Engineering* 15, 113-124 (2005). – Impact factor: 0.922. Times cited: 10 (according to Scopus).

6. C.K. Chua, K.F. Leong, K.H. Tan, F.E. Wiria, C.M. Cheah. “Development of Tissue Engineering Scaffolds Using Selective Laser Sintering of Polyvinyl Alcohol/Hydroxyapatite Biocomposite for Craniofacial and Joint Defects”. *Journal of Materials Science: Materials in Medicine* 15, 1113-1121 (2004). – Impact factor: 1.562. Times cited: 15 (according to Scopus).

International Conference Papers:

1. F.E. Wiria, C.K. Chua, K.F. Leong, M. Chandrasekaran, M.W. Lee. “Modeling of Selective Laser Sintering Heat Transfer Phenomena for Powder-Based Biomaterials”. In Proceedings of the 2nd International Conference on Advanced Research in Virtual and Rapid Prototyping, London: Taylor & Francis, p. 509-512, 28 September – 1 October 2005, Leiria, Portugal.
2. F.E. Wiria, C.K. Chua, M. Chandrasekaran, K.F. Leong. “Theoretical Treatment and Experimental Verification of Selective Laser Sintered Biopolymer Powders”. In Proceedings of the 3rd International Conference on Materials Processing for Properties and Performance (MP³), p. 373-378, 24-26 November 2004, Singapore.
3. C.K. Chua, K.F. Leong, F.E. Wiria, K.H. Tan, M. Chandrasekaran. “Fabrication of Poly(Vinyl Alcohol)/Hydroxyapatite BioComposites Utilizing Rapid Prototyping Implementation in Tissue Engineering”. In Proceedings of International Conference on Competitive Manufacturing (COMA '04), p. 229-234, 4-6 February 2004, Stellenbosch, South Africa.
4. K.H. Tan, C.K. Chua, K.F. Leong, C.M. Cheah, W.S. Gui, W.S. Tan, F.E. Wiria. “Selective Laser Sintering of Biocompatible Polymers for Applications in Tissue Engineering”. In Proceedings of Second International Conference on New Biomedical Materials, 5-8 April 2003, Cardiff, Wales, UK.
5. C.K. Chua, K.H. Tan, K.F. Leong, F.E. Wiria, C.M. Cheah, P. Cheang, M.S. Abu Bakar. “Solid Freeform Fabrication of Polyvinyl Alcohol / Hydroxyapatite Composites for Biomedical Applications”. Presented at Second International Conference on New Biomedical Materials, 5-8 April 2003, Cardiff, Wales, UK.

Manuscripts that are currently have been submitted or being prepared for publication based on this work are listed as follows:

- F.E. Wiria, C.K. Chua, K.F. Leong, M. Chandrasekaran, M.W. Lee. “Theoretical Study of Heat Transfer Phenomena in Selective Laser Sintering for Biopolymer Application”. *Submitted to Acta Biomaterialia*.
- F.E. Wiria, C.K. Chua, K.F. Leong, M. Chandrasekaran, M.W. Lee. “Study of Osteoblast-like Saos-2 Cells on Polymeric Sintered Scaffold”. Targeted journal: *Journal of Biomedical Materials Research – Part A*.
- F.E. Wiria, C.K. Chua, K.F. Leong, M. Chandrasekaran, M.W. Lee. “Behaviour of Saos-2 Cells on Polymeric Sintered Scaffold with Incorporation of Hydroxyapatite”. Targeted journal: *Biomaterials*.

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APPENDIX

Appendix A – MATLAB Program for Temperature Distribution Prediction

```

clear
clc
clf
% qo=1.03e8;
qo=3.17e7;
% qo=2.38e7;
k=2; alpha=9.26e-7; tau=0.0002;
%====Time input====%
to=0.001:0.001:0.01025;

%=====
==%
N=50;
Nt=size(to,2);
for j=1:Nt
    t(j)=to(j);
    for i=1:N
        z(i)=0.0004*(i-1)/N;
        x(i)=z(i)/sqrt(4*alpha*t(j));
        y(i)=z(i)/sqrt(4*alpha*(t(j)-tau));
        ierfc1(i)=(1/sqrt(pi))*exp(-x(i)^2)-x(i)*erfc(x(i));
        ierfc2(i)=(1/sqrt(pi))*exp(-y(i)^2)-y(i)*erfc(y(i));
        T(i)=25+(qo/k)*(sqrt(4*alpha*t(j))*ierfc1(i)-sqrt(4*alpha*(t(j)-tau))*ierfc2(i));
    end
    plot(z,T); hold on
end
end

```


Appendix B – FTIR Wavelength of PVA Specific Chemical Bonds

<i>Type of Bond</i>	<i>Wavelength (cm^{-1})</i>	
	As received PVA	Sintered PVA
<i>C – H</i> (Alkenes)	2941.4	2941.1
	2909.6	2909.6
	1457.2	1457.2
	918.1	918.1
	848.6	849.6
<i>C – O</i> (Alcohol)	1238.1	1283.3
	1144.7	1144.7
	1095.5	1096.5
<i>O – H</i> (Alcohol)	3392.8	3380.2
	1338.8	1331.8
<i>C = C</i> (Alkanes)	1635.6	1653.3