IMPROVING THE MECHANICAL AND FUNCTIONAL PERFORMANCE OF EXTRUSION-BASED ADDITIVE MANUFACTURED SCAFFOLDS FOR BONE TISSUE ENGINEERING

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Appendix A-1
Summary

Scaffold-based tissue engineering (TE) aims to aid in the repair and regeneration of bone defects. Bone defects due to high energy trauma, bone resections, congenital malfunction and severe non-union fractures require a bone substitute for regeneration. At present, the demand of most commonly used bone substitute autogenous cancellous bone grafts far exceeds the supply. Moreover, it is not an ideal solution due temporary disruption of donor site bone structure and considerable donor site morbidity associated with the harvest. Hence, the development of new synthetic bone substitutes or scaffolds that could be used instead of autogenous cancellous bone grafts has become a key priority in bone TE.

In scaffold-based bone TE the scaffold acts as a platform to carry cells or therapeutic agents for regenerative therapies. An ideal scaffold is required to mimic the mechanical and biochemical properties of the native tissue. To effectively achieve these properties, a scaffold should be mechanically robust with suitable architectural qualities to favour flow transport of nutrient for cell growth. It should also have osteoconductive properties to support cells through suitable surface chemistry. In this context high performance extrusion based additive manufactured scaffolds were developed for bone tissue engineering by improving their mechanical, biochemical and cell seeding efficiency.

Mechanical properties of the polymeric/ceramic scaffolds were improved by enhancing the interfacial interaction between the polymeric and ceramic phase through the use of coupling agents. Two different coupling agents, namely silane and POSS
have been used in this research project. The main idea of using a coupling agent was to improve the interfacial interaction between the ceramic and polymer phase. Both of the developed silanized poly (ε-caprolactone)/tricalcium phosphate (PCL/TCP) and POSS modified PCL/TCP scaffolds have significantly improved mechanical properties and are suitable to use for cancellous bone tissue engineering. No detrimental effect of silane modification was found on cells. On the other hand POSS modified scaffolds showed better proliferative capability compared to control PCL/TCP scaffolds, which is due to the exposed TCP on the POSS modified PCL/TCP scaffolds.

To improve the proliferative and osteoconductive properties of the developed silanized PCL/TCP scaffolds, a thin layer of carbonated hydroxyapatite (CHA)-gelatin composite was coated onto the scaffolds by biomimetic co-precipitation process. *In vitro* studies showed promising results of the biomimetic composite coated scaffolds on proliferation and osteogenic differentiation of porcine bone marrow stromal cells. *In vivo* study was also conducted to evaluate the performance of biomimetic composite coated samples.

To improve the functional performance of developed POSS modified PCL/TCP scaffolds by providing a cell entrapment system, a novel hierarchical scaffold that combines the advantageous properties of AM scaffold and porous foam scaffold was developed. In the hierarchical structure PCL/TCP(POSS)-foam scaffolds the macrosized PCL/TCP(POSS) filaments provide mechanical support and the porous gelatin foam structure formed by freeze drying acts as a cell entrapment system. From the manufacturing point of view, to fabricate hierarchical scaffolds, our developed approach is considerably simpler than combining electrospinning with AM. *In vitro*
results showed notably higher proliferative capability on PCL/TCP(POSS)-foam scaffolds compared to PCL/TCP(POSS) scaffolds.

In summary, it has been found that coupling agents improve mechanical properties of the polymer/ceramic scaffolds significantly. Scaffolds with improved mechanical properties can be further modified to enhance functional performance of the scaffolds. This study will make a significant contribution in the field of extrusion based AM scaffolds by improving mechanical properties of the scaffolds by using coupling agents, and functional performance of the scaffolds by developing thin biomimetic composite coating and hierarchical structure for cells entrapment system.
Abbreviations

3DP - Three dimensional printing

AM - Additive manufacturing

BMP - Bone morphogenic protein

BMSC - Bone marrow stromal cells

DMEM - Dulbecco’s modified Eagle’s medium

CAD - Computer aided design

CHA - Carbonated hydroxyapatite

CT - Computed tomography

EDC - 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

FBS - Fetal bovine serum

FD - Filament distance

FDA - Food and Drug Administration

FDA - Fluorescein diacetate

FDM - Fused deposition modeling

FG - Fill gap
FTIR - Fourier transform infrared spectroscopy

GPTMS - 3-glycidoxypropyl trimethoxysilane

HA - Hydroxyapatite

LG - Layer gap

LTDM - Low temperature deposition modeling

MRI - Magnetic resonance imaging

NHS - N-hydroxysuccinimide

SEM - Scanning electron microscopy

TE - Tissue engineering

PBS - Phosphate buffer saline

PCL - Poly (ε-caprolactone)

PEEK - Polyetheretherketone

PEG - Poly (ethylene glycol)

PGA - Poly(glycolic acid)

PHBV - Poly(hydroxybutyrate-cohydroxyvalerate)

PI - Propidium iodide

PPF - Poly (propylene fumarate)
Abbreviations

PLA - Poly(lactic acid)

PLGA - L,L-lactide-co-glycolide)

RW - Road width

SBF - Simulated body fluid

SES - Screw extrusion system

SLA - Stereolithography apparatus

SLS - Selective laser sintering

ST - Slice thickness

TCP - Tri calcium phosphate

XPS - X-ray photo electron spectroscopy
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Chapter 1

Introduction

1.1 Background

The term tissue engineering (TE) is used to mean the replacement, repair, and/or regeneration of biological tissues and organs. This term was “coined” at a committee meeting at the National Science Foundation during the fall of 1987. This committee meeting led to the first congress called “tissue engineering,” which was held in early 1988 at Lake Tahoe, California [1]. TE is an exciting idea which has experienced an explosive growth. It is an interdisciplinary field which combines the basis of life sciences and medicine with the methods of engineering. The purpose of TE is to develop a biological substitute that can replace, repair and/or improve tissue function.

Scaffold-based tissue engineering (TE) aims to aid in the repair and regeneration of bone defects. Though bone is a highly regenerative organ, large bone defects due to high energy trauma, bone resections, congenital malfunction and severe non-union fractures require a bone substitute for regeneration [2]. In this aspect, the most practised solution is autografting which may not be very ideal due to its short supply, temporary disruption of donor site bone structure and considerable donor site morbidity associated with the harvest [3, 4]. Therefore, the development of new synthetic bone substitutes or scaffolds that could be used instead of autogenous cancellous bone grafts has become a key priority in bone TE [5-7].

In scaffold-based bone TE the scaffold acts as a platform to carry cells or therapeutic agents for regenerative therapies. An ideal scaffold is required to mimic the
mechanical and biochemical properties of the native tissue. To effectively achieve these properties, a scaffold should be mechanically robust with suitable architectural qualities to favour flow transport of nutrient for cell growth. It should also have osteoconductive properties to support cells through suitable surface chemistry [6].

Additive manufacturing (AM) technologies are fast becoming the technologies of choice for fabricating scaffolds for bone TE due to reliability, high degree of reproducibility and the potential to overcome the limitations of conventional manual-based fabrication techniques [8]. Complex scaffold architecture designs based on a hierarchical approach can be readily fabricated through AM [9, 10]. Screw extrusion system (SES) which is a very promising AM technique has been used to fabricate both polymer and polymer/ceramic composite scaffolds with honeycomb like structure [11, 12]. However, the focuses of the studies related to additive manufactured bone TE scaffolds are mostly limited to explore different fabrication techniques, optimize suitable porosity and pore size of scaffolds, and use of Ca-P type ceramics into polymer matrix to get improved mechanical properties. Hence, an important issue which has not yet been studied critically is using coupling agent to improve the mechanical properties of the composite scaffolds in context of bone TE. Moreover, different surface modification techniques to improve the proliferative and osteoconductive properties of the additive manufactured scaffolds are still in its infancy.

1.2 Challenges in additive manufactured bone TE scaffolds

In scaffold-based bone TE an ideal scaffold should be mechanically stable; that is the mechanical properties of the scaffold must be sufficient to withstand \textit{in vivo} stress and loading [13] and the choice of material should have a degradation and resorption rate
so that the scaffold is retained until the fracture is remodelled by the host tissue [6]. Specially, it was proposed that the implanted scaffolds should match the stiffness and strength of native tissue at the time of implantation [6]. The scaffolds should also be biocompatible; that is the scaffolds must not evoke cytotoxicity and/or unresolved inflammatory response [6]. Moreover, it is desirable that the scaffolds should also be osteoconductive. Other highly desirable features concerning the scaffold are its controllable interconnected porosity and vascularization. Therefore, both the mechanical properties as well as the biochemical properties like biocompatibility and osteoconductivity of the scaffolds need to be considered to design and fabricate “potential” scaffolds for bone TE.

To improve the mechanical properties of the polymeric scaffolds, ceramic particles have been mixed into the polymer matrix directly [12, 14]. However, only a slight improvement in compressive modulus and compressive strength was achieved with polymer/ceramic composite scaffolds compared with those of polymer scaffolds [14]. As reported recently in the review paper by Rezwan et al. [15] the limited improvement on mechanical properties of polymer/ceramic composite scaffolds is because of the relatively low interfacial bonding between ceramic particles and polymer matrix. Hence, using coupling agent to improve the interfacial bonding ceramic particles and polymer matrix which has been neglected in context of bone TE should be addressed with priority.

Though there are many advantages of AM scaffolds, it has some inherent limitations. For example, the surfaces of the extrusion based AM scaffolds are generally smooth and hence it is difficult to coat with thin biomimetic composite within a short period of time. As a consequence, any efficient biomimetic coating approach for AM
scaffolds has not yet been reported. Moreover, the cell seeding efficiency of AM scaffolds are poor. To improve the cell seeding efficiency a combinational approach by combining both AM and electrospinning has been introduced. Though the hybrid scaffolds resulted by AM technology with electrospinning are promising, more efforts are needed in the insulation of the AM robot from the electrospinning high voltage collector [16]. Therefore, a simple combinational approach should be developed to improve the cell seeding efficiency, and thereby to improve the functional performance of the scaffolds. The hypothesis, objectives and significance of this research will be elaborated in the following sections.

1.3 Research hypothesis and objectives

Having the above mentioned challenges in mind the following hypothesis can be formed:

“To further improve the range of bone TE applications we need to improve the mechanical and functional performance of extrusion-based additive manufactured scaffolds.”

The main objective of this study was therefore to develop additive manufactured scaffolds with improved mechanical and functional performance for bone TE applications. In this context, a number of objectives that have been set to accomplish the principal aim are as follows:

- Evaluate the suitability of in-house screw extrusion based AM technology to fabricate polymer-ceramic composite scaffolds for bone TE.
Modify polymer/ceramic composite with different coupling agents, and then evaluate the developed composite by fabricating extrusion-based AM scaffolds in terms of mechanical properties and biocompatibility.

Improve the proliferative and osteoconductive properties of the developed scaffolds by giving composite coating through biomimetic co-precipitation process.

Develop a combinational approach to improve the cell seeding efficiency of the developed scaffolds.

Evaluate the biomimetic composite coated scaffolds in rat calvarial defect.

1.4 Significance of the research

The results of this present study may have significant impact on the application of scaffolds in bone TE application by providing additive manufactured scaffolds with improved mechanical and functional performance. The results may particularly contribute to the better understanding of the effects of different coupling agents on polymer/ceramic composites for bone TE application. Furthermore, the results may extend the understanding of the basic principles to give effective biomimetic coating on additive manufactured scaffolds and also different ways to improve functional performance of additive manufactured scaffolds for bone TE.

In this research poly (ε-caprolactone)/tricalcium phosphate (PCL/TCP) has been taken as a benchmark in the field of additive manufactured bone TE scaffolds. The reason of the huge popularity of PCL in the field of biomaterials is due to its versatility in applications ranging from sutures to wound dressings, artificial blood vessels, nerve
regeneration, drug-delivery devices and bone engineering applications. Many PCL based
drug-delivery and medical devices have FDA approval and CE Mark registration.
Another important feature of PCL is it does not have any acidic degradation like
poly(lactic acid) (PLA) and poly(lactic-co-glycolide) (PLGA). However, it is hoped that
the developed methods during this research could be translated to other aliphatic
polyester based polymer like poly (lactic acid) (PLA) and poly (lactic-co-glycolide)
(PLGA) if needed.

1.5 Structure of thesis

There are eight chapters and one appendice in this thesis. This chapter started with
the importance and present challenges of additive manufactured scaffolds for bone TE,
and ends up with the hypothesis, aim and significance of this research work.

Chapter 2 presents a comprehensive review on additive manufactured scaffolds for
bone TE by describing design, materials and fabrication technologies used for scaffolds.
Moreover, the recent challenges with additive manufactured scaffolds for bone TE is
highlighted with simplicity.

Chapter 3 describes the main features and interdependence of the different process
parameters of in house SES. The methodological evaluation of this in house SES
fabricated scaffolds is also discussed.

Chapter 4 presents a comprehensive study of using different coupling agents to
enhance the mechanical properties of the scaffolds.

Chapter 5 describes and evaluates a biomimetic composite coating process on
previously developed additive manufactured scaffolds.
Chapter 6 describes a combinational approach by combining AM and freeze drying to improve the cell seeding efficiency of the scaffolds.

Chapter 7 evaluates the performance of the biomimetic composite coated additive manufactured scaffolds in rat calvarial defect.

Chapter 8 consists of conclusions and contributions of the research work. In addition, some directions for future work related to this study are also given.
Chapter 2

Literature Review

2.1 Introduction

Many patients who are in need of organ transplantation suffer greatly due to the lack of suitable donors. At present, the demand for replacement organs far exceeds the supply. In addition, many bone grafting procedures are carried out worldwide on a daily basis, which although effective, are not ideal solutions. Scaffold-based tissue engineering (TE) aims to aid in the repair and regeneration of bone defects. Using this approach, the scaffold acts as a platform which carries cells or therapeutic agents for regenerative therapies. To achieve this, a scaffold should have some desirable properties. In general, the most important thing is biocompatibility; that is the scaffolds must not evoke cytotoxicity or unresolved inflammatory response [6]. In addition, it should be mechanically stable; that is the mechanical properties of the scaffold must be sufficient to withstand in vivo stress and loading [13] and the choice of material should have a degradation and resorption rate so that the scaffold is retained until the fracture is remodelled by the host tissue [6]. Specially, it was proposed that the implanted scaffolds should match the stiffness and strength of native tissue at the time of implantation [6]. Other highly desirable features concerning the scaffold are its controllable interconnected porosity and vascularization.

This chapter aims to identify the state-of-the art and future direction of additive manufactured bone TE scaffold. The emphasis will be on the design, material and additive fabrication of the bone TE scaffolds.
2.2 Background of scaffold technology

When we face the failure of a vital organ, we may need replacement. The replacement can be a transplant from another person or it can be an artificially produced organ. If we are unable to find a replacement then it may cause severe disability or even death. However, the use of transplantation faces problems like immunorejection and currently the demand for organs far exceeds the supply. Therefore, the necessity of creating artificial organs in the laboratory increases day by day. Though there are still many years to go before a doctor can order a ready-made organ, with the advancement in TE or regenerative medicine and biomanufacturing, we should eventually be able to achieve the “functional spare part”.

TE is an interdisciplinary field. It combines the basis of life sciences and medicine with the methods of engineering. “The goal of TE is to go beyond the limitations of conventional treatments based on organ transplantation and biomaterial implantation [17]”. TE has three basic components and these are the cells, scaffolds and signals. From Figure 2.1 it can be seen that cells are collected from patients (they can also be collected from some other persons) and then cultured in a 3D environment. Sometimes a suitable environment is provided through bioreactors. After a certain period scaffolds are transplanted to the desired location.

The therapy of TE can be done with the cultivated cell or it can be done by production of tissue constructs. To create tissues in vitro, the respective cells have to be settled on a natural or artificial extra cellular matrix (ECM). This construct is called a scaffold. Study into scaffolds is currently one of the important issues in TE. Scaffolds act as a temporary support for cells to attach, proliferate and differentiate. They coordinate the growth of tissues in a desired shape. The scaffolds are then cultured with
cells, which are grown in a controlled environment. Figure 2.2 illustrates the interdependency of three broad areas of scaffold technology; design, biomaterials and fabrication process. All these three areas have simultaneous contribution to scaffold technology.

![Figure 2.1 Primary considerations for TE.](image)

![Figure 2.2 Block diagram of the main areas of the scaffold technology.](image)
2.3 Design of scaffolds

2.3.1 Porosity and pore size of scaffolds

Porosity and pore size are two important structural features of scaffolds that have considerable effect on cell growth. The pores of the scaffold should be connected to promote nutrient supply and tissue growth. In vitro, lower porosity stimulates osteogenesis, whereas, in vivo higher porosity and pore size result in greater bone ingrowth [18]. Different types of cells need different pore sizes for optimum growth and it was found that chondrocytes and osteoblast type cells grow better within the pore size of 380-405 μm [19]. Works related to porosity and pore size are comprehensively reviewed by Karageorgiou et al. [20]. They have noted that 100 μm pore size is essential for cell migration and transport, and a recommended pore size of greater than 300 μm is better for increasing the formation of new bone and capillaries. The reason appears to be that osteogenesis progression is affected by pore size. Small pores induce osteochondral formation before osteogenesis, whilst large pores that are well vascularized induce direct osteogenesis.

Tissue forms quickly on the outer surfaces of the scaffold and this phenomenon hinders cell penetration and nutrient exchange to the scaffold core. This problem can be addressed by building scaffolds with both random and anisotropic open porous structures. Graded porosity has its importance in multiple tissue interface regions such as in articular cartilage/bone transplant [21]. Figure 2.3(a) shows a gradient porosity scaffolds by varying different filament to filament distance while keeping the lay down pattern constant [22]. Biphasic scaffolds showed potential in the repair of large osteochondral defect [23]. In this perspective, we are proposing a novel functionally
graded hybrid scaffolds. With this concept it would be possible to tailor both bone and cartilage phases into one implant. Therefore, it will reduce the complexity in handling and the surgery will be more efficient. Moreover, this multi-phasic constructs will provide new strategies for tissue repair. The schematic of the hybrid construct is given in Figure 2.3(b).

Figure 2.3 Graded scaffolds (a) with controlled porosity with varying pore geometry via FDM [8]; (b) proposed hybrid scaffolds by our group, where zone i: bone phase, PCL/20%TCP with 65-70% porosity; zone ii: bone plate phase, PCL/20%TCP with 30-40% porosity; zone iii: calcified cartilage phase, PCL/10%TCP with 70-75% porosity and zone iv: cartilage phase, PCL with 70-75% porosity.

2.3.2 Architecture of scaffolds

Although AM scaffolds can be 100% interconnected and highly porous with very regular structure, previous study also reported less cells and tissue penetration within interior of the additive manufactured scaffolds [14]. Figure 2.4(A) shows the traditional structure fabricated by extrusion based AM technology. At the middle of this structure cells grow in a limited manner or even die during cell culture due to the lack of nutrient supply. To solve this problem we have proposed the structure having a through hole for nutrient supply as shown in Figure 2.4 (B, C). As well as possibly improving the mechanical properties of a scaffold, it is conjectured that through-holes may also become useful to increase the mass transport of oxygen and nutrients deep within and removal of waste products from the scaffolds. This sealed hole can perhaps even be used for storage.
of nutrients or growth factors. Some recent studies have also introduced through holes in scaffolds fabricated using other AM technologies like 3-dimensional printing (3DP) [24] and stereolithography (SLA) [25] to improve the mass transport of oxygen and nutrients. However, validation of the usefulness of these through holes or macro channels during in vitro study remains pending.

![Figure 2.4 Schematic of (a) traditional scaffold fabricated by FDM type system, (b) structure proposed by us for channeling and (c) SEM images of the structure for channeling or storage of nutrients [88].](image)

Architecture of the AM scaffolds has also been designed to improve the cell seeding efficiency of the scaffolds. In extrusion based scaffolds different lay down patterns of the extruded filaments were used to improve the cell seeding efficiency of the scaffolds [26]. By varying the angle of the lay down pattern in successive layers a spirally convoluted porous scaffold can be fabricated [22]. Scaffolds designed with inclined layers of 45° have been fabricated (as shown in Figure 2.5) using 3DP to improve the cell seeding efficiency [27]. Eventually, this design enhanced cell attachment because the cells are hindered from sliding down the structure during static cell seeding process. These scaffolds showed good cell proliferation into the inside of the structure without clogging.
Different structures of scaffolds affect the dynamic mechanical properties of scaffolds. It has been reported that fiber deposition and orientation in scaffold has influence on their viscoelastic properties [28]. With increase of porosity by changing fiber diameter, spacing, orientation and the layer thickness, viscous parameters like damping factor, creep and unrecovered strain are affected.

2.4 Materials of scaffolds

2.4.1 Bioactive ceramic phases

Different biomaterials like bioactive ceramics, biodegradable polymers and most importantly polymer-ceramic composites have been used to fabricate bone TE scaffolds. Most commonly used bioactive ceramics in the field of AM are calcium phosphates like hydroxyapatite (HA) and tricalcium phosphate (TCP). HA is the most important inorganic constituent of natural hard tissue [29] and therefore it is obvious why HA and related calcium phosphates like TCP have been studied as an important constituent of scaffold materials. Different in vitro and in vivo studies showed that calcium phosphates support cells attachment, proliferation and differentiation irrespective of form and phase [15]. Usually calcium phosphates are stable and have an excellent biocompatibility with minimal immunologic and foreign body reactions [30]. HA scaffolds with controlled
internal structures and high resolution were fabricated using 3DP [27, 31]. *In vitro* results showed that the cells proliferated deep into the fabricated scaffolds proving the potential of 3D printed HA scaffolds. HA scaffolds fabricated through FDM has also shown good biocompatibility as indicated by a dense covering of human osteoblast sarcoma cells after 48 hr of *in vitro* study [32]. Indirect fabrication using casting in AM moulds provides another way to fabricate ceramic scaffolds with controlled structure [33, 34]. However, the indirect fabrication has the shortcoming of increased scaffold fabrication time compared with direct methods.

At present the use of pure HA and TCP scaffolds are limited especially for the applications involving large skeletal defects because of their brittleness and propensity to fracture. Eventually the ceramic scaffolds failed to combine good mechanical properties with an open porosity [18], which restricted its use from medium to high compressive loading.

### 2.4.2 Biodegradable polymer matrices

There are two types of biodegradable polymers: natural polymer and synthetic polymer. Natural polymer includes polysaccharides (starch, alginate, chitin, hyaluronic acid derivatives) and proteins (soy, collagen, fibrin gels). Among these natural polymers chitosan, collagen, gelatin and starch have been used successfully to fabricate AM bone tissue engineered scaffolds [35-40]. Chitosan is deacetylated derivative of chitin, the second most abundant natural polymer after cellulose, commonly found in shells of crustaceans and cell walls of fungi [41]. Interesting properties that enable the linear polysaccharide chitosan as a bone scaffold material are its unique cationicity, intrinsic antibacterial nature, minimal foreign body reaction and suitable gelling properties [41-
Geng et al. [38] have fabricated porous chitosan scaffolds using an extrusion-based AM technology, which sequentially dispenses sodium hydroxide solution, and chitosan dissolved in acetic acid resulting in gel-like chitosan scaffolds. To get hydrated scaffolds first the scaffolds were thoroughly washed, heated at 40 ºC followed by freeze drying. The fabricated chitosan scaffolds showed good biocompatibility. Porous structure prepared by functionalized photo curable chitosan showed very good endochondral ossification upon implantation [39]. In line with this development of functionalizing natural polymers, a successful use of photo curable gelatin to fabricate scaffolds made by SLA should be noted [37]. It should be mentioned here that collagen-based gelatin has many of biological functional groups, and as gelatin is denatured, it overcomes the risk of immunogenicity that is associated with collagen. In vitro cell culture of methacrylate functionalized gelatin scaffolds reported no cytotoxicity which signifies the biocompatibility of developed monomer. Lam et al. were the first group to demonstrate the use of natural polymers to fabricate scaffolds by 3DP [40]. They have used starch based polymer powder (cornstarch, dextran and gelatin) as the scaffold material and water as the binder. Starch and dextran, which are both polysaccharides, have been shown to be biocompatible [40, 45]. On the other hand using water as the binder eliminates the risk involved in the residual solvent and also opens up the possibility of using biological agent during the scaffolds fabrication process by 3DP.

The most widely used biodegradable synthetic polymers from the past decade for bone TE scaffolds are saturated poly-α-hydroxy esters, including poly(lactic acid) (PLA) and poly(glycolic acid) (PGA), as well as poly(lactic-coglycolide) (PLGA) copolymers [15]. These polymers are easily processable into a variety of architectures and shapes with a wide range of tailored mechanical and degradation properties by using the monomer ratios in lactide/glycolide copolymers [6]. PLLA scaffolds with degradation
rate appropriate for bone repair has been fabricated through extrusion based AM technology [46]. However, the disadvantage of polylactone type polymer is accumulation of acidic matter while degrading, and thus the abrupt release of these acidic degradation products can be a cause of inflammation of the surrounding tissue [47, 48].

PCL is another important aliphatic polyester which possesses superior rheological and viscoelastic properties over many of its resorbable-polymer counterparts, and hence it is very easy to fabricate and manipulate into a large range of AM bone TE scaffolds [49-51]. At National University of Singapore researchers have developed and patented the parameters necessary to process medical grade PCL by FDM. These scaffolds have been extensively studied clinically for more than 5 years and gained Food and Drug Administration (FDA) approval in 2006 [49, 52]. However, PCL is hydrophilic and its degradation rate is very slow. To vary the ratio of hydrophobic/hydrophilic constituents by copolymerization and to modulate degradability polyester-polyether block co-polymers composed of PCL or PLA and poly (ethylene glycol) (PEG) have been studied [53-55]. To enhance hydrophilicity and control degradability PEG and PLA has been added into PCL main chain, respectively. Study demonstrated that PEG-PCL-PLA co-polymer is suitable for fabricating scaffolds with honeycomb like structure having completely interconnected and controlled pore channels by extrusion based AM technology. *In vitro* cell study using primary human fibroblasts also showed biocompatibility of the developed co-polymer [54].
2.4.3 Polymer/ceramic composite scaffolds

Recently composite materials have been investigated comprehensively as they have the potential to harness the advantageous properties of both polymer and ceramics (mainly Ca-P), and thereby improve the biochemical and mechanical properties of the bone tissue engineered AM scaffolds [12, 14, 15, 18, 56-61]. Polymer/ceramic composite scaffolds showed more apatite coating formation [62] and improved cellular response [12] compared to polymeric scaffolds. Polymer/ceramic composite scaffolds also showed more hydrophilicity during contact angle measurements [14] and favourable degradation and resorption kinetics compared to polymeric scaffolds. Incorporation of Ca-P based ceramic into the fast degrading aliphatic polyester like poly (L,L-lactide-co-glycolide) (PLGA) helps to neutralize the acidic resorption by-product of the polyester, and thus aids to circumvent the formation of harsh environment for the cells due to decreased pH [63].

Polymer/Ca-P composite scaffolds have improved mechanical properties as it improves strength via the ceramic phase and toughness and plasticity via the polymer phase. However, a crucial aspect is the interfacial bonding between the ceramic and polymer phase which has been ignored in the development process of composite material for bone TE scaffolds and therefore the improvement on mechanical properties of polymer/ceramic scaffolds is limited [15, 18]. The particle size of Ca-P and distribution in the polymer matrix have been identified as important aspects to improve mechanical properties [64]. Apparently, an increased volume fraction of the Ca-P filler into the polymer matrix also leads to stiffer scaffolds [61]. However, during mechanical loading failure can preferentially occur at the interface of ceramic and polymer matrix.
Therefore, improving the interfacial bonding between the ceramic and polymer phase to improve the mechanical properties of the scaffolds are imperative.

### 2.5 Fabrication of scaffolds

#### 2.5.1 Conventional fabrication techniques

The basis of conventional scaffold fabrication techniques are textile and polymer processing technologies. Conventional fabrication techniques include solvent casting particulate leaching, gas foaming, fiber meshes/fiber bonding, phase separation, melt molting, freeze drying and solution casting [8]. Each of these techniques has limitations to precisely control pore size, pore geometry, pore interconnectivity and spatial distribution of pores. These techniques are mostly manual, labor intensive and time consuming, which results in inconsistent macro and micro structure. This could lead to partially connected pathways, which hampers the supply of nutrients and the ingrowth of tissue into the scaffold [65]. Besides these limitations, most of the conventional techniques usually involve extensive use of toxic organic solvents in order to convert raw material into the final scaffolds. Residual solvents in the scaffolds especially in thicker constructs lead to detrimental effect on cells and / or nearby tissues [8]. In this context additive manufacturing technology which is a computer controlled fabrication process, reveals a great potential by offering design dependent scaffolds, as opposed to the process dependent scaffolds offered by conventional techniques [8, 9, 65-70].

#### 2.5.2 Additive manufacturing techniques

Additive manufacturing refers to a group of techniques that can generate a physical model in a layer-by-layer manner directly from computer aided design data. Over the
past two decades more than 20 additive manufactured techniques have been developed with an effort focused on the rapid manufacturing of prototypes for non-biomedical applications, and these techniques separate themselves mainly by the method by which the layers are laid down, solidified and attached to the previous one [8, 9, 67]. Though there are variants in additive manufacturing based on the way they build up the 3D model, they all have three basic steps: data input, data file preparation and structure building. In particular in a medical context, for a patient specific physical model, the input data is captured by computed tomography (CT) or magnetic resonance imaging (MRI) scans and this patient specific data is used to generate a computer based 3D model by computer aided design (CAD) software. This 3D CAD model is then imported into AM system software to be sliced into thin horizontal layers with specified tool paths for each layer. Successively the data are implemented by the additive manufacturing machine that creates the customized physical model. Today AM technologies have been used extensively to fabricate bone tissue engineering scaffolds with customized external shape and predefined internal morphology with full control of pore size and pore distribution. Some important AM techniques include stereolithography apparatus (SLA), selective laser sintering (SLS), 3D printing and extrusion based processes, which will be discussed in the following sections.

2.5.2.1 Stereolithography apparatus (SLA)

SLA was the first commercially available AM system introduced by 3D System in 1986. (www.3dsystems.com). The manufacturing of 3D objects by SLA is based on selective polymerization of a liquid photo curable monomer by an ultraviolet laser beam. With the computer guided laser beam a pattern is illuminated on the surface of liquid photo curable monomer in accordance with the CAD data. As a result of this
illumination, polymerization occurs and the liquid solidifies to a certain depth. After the first layer is built, the platform holding the model is moved down from the surface into the vat and the built layer is submersed with liquid resin. A pattern is then cured in this second layer. The procedure is repeated until the model is completed. After that the model is removed from the platform, washed of excess resin and cured in a UV oven and finished by smoothing the surface irregularities. SLA has the highest accuracy and resolution among all the AM technologies which can build objects that measure several cubic centimetres at an accuracy of 20 μm, whereas most other AM techniques build objects with an accuracy of 50-200 μm in size [71]. However, to date the use of SLA in biomedical industry is mainly focused on building anatomical models for surgical planning [72].

Poly (propylene fumarate) (PPF) is the most comprehensively studied biodegradable and photo-crosslinkable polymers [73] because of its convenient photo-crosslinking characteristics and suitable mechanical properties after UV crosslinking. Composite structure can also be fabricated using SLA. PPF-HA composite structures was fabricated by mixing PPF and HA particles in diethylfumarate (DEF) [74]. Protein binding molecules biotin has also been immobilized by excimer laser photobiotin. Furthermore, HA scaffolds with well-defined channel size and desired connection pattern can be fabricated with indirect SLA technique [33]. Eventually this is a lost-mold technique where SLA was used to create negative molds and a highly loaded curable HA suspension was used as the ceramic carrier. Most interestingly some recent studies showed promising results on synthesizing a series of photocurable natural polymers, which eventually extends the use of natural polymer in SLA techniques [37, 39].
Despite these achievements, till today, the limited number of commercially available resins can be considered as the main limitation of SLA technique. Moreover, the technique is still limited to the use of a single resin at a time, whereas 3D printing and extrusion based system allow the fabrication of scaffolds with different materials simultaneously by using multiple cartridges [71].

2.5.2.2 Selective laser sintering (SLS)

SLS technique normally uses a CO₂ laser beam to selectively sinter thin layers of powdered materials following the cross-sectional profiles carried by the slice data to form 3D objects. The interaction of the laser beam with the powder raises the powder temperature to its glass transition temperature, which causes particles to fuse together in a small layer. After finishing each layer a new layer of powder is supplied using a mechanical roller. Each new layer bonds to those previously sintered layers also because of the sintering process [75].

SLS showed great potential in producing complex porous ceramic matrices suitable for implantation in a bone defect [76, 77]. However, theoretically SLS is suitable to use any powdered biomaterial which fuses but not decomposes under a laser beam. Powder blends of polyetheretherketone (PEEK) and PEEK-HA has been sintered on SLS by varying various process parameters like the part bed temperature, laser power and scan speed [78]. This study showed promising results on laser sintering a high melting point unconventional biopolymer PEEK in lower temperature environment and demonstrated that a low part bed temperature should be complemented by a higher laser power. This study also demonstrated that the wt% of HA in PEEK should not exceed 40% in order to make a structure with good integrity. SLS has also been used to fabricate bioresorbable polymers or composites structures or scaffolds [50, 57-59, 79-82]. Williams et al. have
demonstrated the potential of SLS to fabricated PCL scaffolds [50]. They have designed PCL scaffolds computationally and then fabricated via SLS. The fabricated PCL scaffolds were implanted subcutaneously in mouse with BMP – 7 transduced fibroblasts. The *in-vivo* histological evaluation and micro-computed tomography (mCT) analysis of implanted scaffolds demonstrated the generation of bone. In addition to the fabrication of bioresorbable polymer scaffolds, polymer-ceramic composite scaffolds have also been fabricated [57, 79, 82]. Wiria, F. E. *et al.* [57] have investigated the feasibility of fabricating PCL/HA scaffolds by SLS. They have also investigated the process parameters on the sintering effect of the constructs. By observing the necking, porous network formation and also on the basis of handling stability of the construct they have determined some preferred parameters. They noticed that sintering of the PCL/HA composite is more difficult than sintering PCL alone because of the addition of HA powder, although the sintering result was good.

In SLS process volumetric absorption of the laser radiation by the polymer leads to melting of the whole polymer particle, which eventually decreased the possibility of using bioactive molecule during the fabrication process. To overcome this limitation, a modified SLS process – surface selective laser sintering (SSLS) has been introduced [83]. In this modified process initiation of sintering starts only by melting the surface of polymer particle. To achieve this PLA, which does not absorb near-infrared laser radiation, has been modified with biocompatible carbon particles and only these carbon particles absorb laser radiation which leads to the localized sintering. This “gentle” process improves the probability of incorporating bioactive molecules during the SLS scaffold fabrication process [83].
Despite these advancements and advantages offered by SLS, there are some limitations. For example, SLS fabricated structures are limited to the smaller pore size ranges (<50 μm). Moreover, as this process involves high processing temperature, it is mainly suitable to thermally stable polymers [8].

2.5.2.3 Three-dimensional printing (3DP)

3DP technique was developed at Massachusetts Institute of Technology (MIT) by Sachs et al. in 1993 [84] and today it is a popular technique to fabricate tissue engineering scaffolds. This technique is based on ink jet printing technology for processing powder materials. During fabrication, a printer head is used to deposit or print a stream of binder solution over the surface of a powder bed following the object’s profile as generated by the system computer. After finishing the 2D layer, a fresh layer of powder is laid down. The layers merge together due to the next cycle of binder solution until the whole part is completed. The completed 3D object is collected by removing the unbound powder.

3DP has been used widely to fabricate tissue engineering scaffolds for its simplicity [31, 35, 40, 85-90]. This technique operates at room temperature, and thereby suitable for processing temperature sensitive materials especially natural biopolymers. Lam et al. have demonstrated the feasibility of using natural biopolymers by 3DP with a blend of starch based powder containing cornstarch, dextran and gelatin, bounded by distilled water [40]. It is interesting to mention that as the binder is water, and therefore this process eliminates the problem linked to the use of an organic solvent. However, to enhance the mechanical stability of the scaffolds and also to decrease the solubility in water a long post-processing was necessary. Collagen scaffolds with complex internal morphology and macroscopic shape has also been fabricated by indirect 3DP using a
sacrificial mould [35]. In addition, ceramic scaffolds have successfully been fabricated using 3DP [31]. Ceramic scaffolds fabricated using this technique showed higher compressive strength and larger pores compared to the conventionally sintered calcium polyphosphate scaffolds of similar porosity [90].

Limited pore size (<50 μm) is one of the disadvantage of 3DP. Larger pore can be created in 3DP scaffolds by mixing porogens into the powders prior to the fabrication process [85, 86]. However, incomplete leaching of porogen can lead to residues. Moreover, as 3DP structures are powder supported and become powder filled due to the unbound powder, there is difficulty in removing internal unbound powder.

2.5.2.4 Extrusion based system

In 1992 Crump developed the first commercially available extrusion based system known as fused deposition modeling (FDM) [91]. In this system, a thin thermoplastic filament is melted by heating and the semi molten material comes out through an orifice while it is guided by a robotic device. The extruded material cools, solidifying itself and fixing to the previous layer [92]. Successive layer formation on top of another layer forms the desired 3D porous structure. Porosity of the structure varies in the XY plane and Z direction. The porosity in the XY plane is created by controlling the distance between two adjacent filaments, while porosity in the Z direction mainly governed by the diameter and lay down angle of the extruded filament [67].

FDM has been used extensively to fabricate scaffolds with a reasonable range of channel size, filament diameter and porosity [93, 94]. Highly interconnected and reproducible bioresorbable structure can be fabricated with FDM. However, the commercial FDM system requires the material in the form of filaments with specific size
Chapter 2.

and material properties to ensure regular material flow and the fabrication of the regular structure. This imposes a limitation on the processing window and the number of materials that can be explored for higher load bearing applications.

To minimize costs and to permit use of a wider range of materials, a compressed-air extrusion system was developed [54, 55] (shown in Figure 2.6(B)). However, in such a system it is difficult to go beyond a certain pressure due to the complexity of the required valve system. As a result, the limited pressure would require materials with suitable melt-flow properties and it may not be possible to create structures suitable for high/medium load bearing applications. Also, the possibility of entrapped air bubbles in the polymer melt may result in imperfect filament deposition and parts with poor mechanical properties [95].

To overcome such disadvantages and to further widen the range of possible materials a screw-extrusion system can be used (as shown in Figure 2.6 (C)). The screw-extrusion system (SES) consists of a robotic-arm, barrel and heater as like the conventional FDM machine. The robotic arm enables movement of the barrel in the x-y-z directions. The heater melts the material, which is then extruded by pressure using a turning screw feed [11, 12, 92, 96]. The parameters which would determine the quality of the extruded scaffolds include the motor speed, dispensing speed, the melt temperature, the platform temperature, layer thickness, nozzle size and road width [92, 95]. In a wider perspective understanding different process parameters relating to the scaffolds fabrication process is very important. Hence, computational studies of different process parameters like melt flow behavior, pore size, porosity of extrusion based system have been done [92, 97, 98].
In the developmental process of extrusion based system combining two extrusion systems can be a potential fabrication technique to use different material from hydrogel to polymeric/ceramic composite. Thus combining two extrusion systems in a single robot facilitates the fabrication of multiple material scaffolds. In this context the Polytechnic Institute of Leiria has developed a variation of FDM consisting of two different extrusion systems; one rotational system for multi material deposition acted by a pneumatic mechanism and another one for a single material deposition system that uses a screw to assist the deposition system [65]. Multiple material scaffolds can also be fabricated with low temperature deposition modeling (LDM) under 0 °C, and this technique fabricates scaffolds with hierarchical macro and micro pores by integrating extrusion and phase separation [99-101]. To fabricate scaffolds through LDM, at first the material was dissolved into solvent at room temperature and then extruded into a low temperature refrigerator to fabricate frozen scaffolds with macropores. After that the scaffolds were freeze dried to evaporate residual solvent which eventually creates micropores.

Extrusion based system has also been using in the area of cell printing. This technique is offering a great potential in the field of organ printing by printing gels,
single cells and cells aggregate [102, 103]. Scaffolds fabricating by extrusion based system has also been using in the field of cancer research [104].

Despite the credibility of extrusion based system, it has some limitations. As an example, the pore openings for the scaffolds are not consistent in all three dimensions. The seeding efficiency of the extrusion based scaffolds is limited. To improve the cell seeding efficiency of the extrusion based scaffolds combinational approaches have been taken, which will be discussed in the following paragraph. Besides these limitations, in-general the extrusion based scaffolds have a smooth surface which is not suitable for initial cell attachment. To overcome this, surface roughness has been incorporated with the aid of a piezoelectric vibration system [105]. In this process well-aligned wavy shaped surface roughness was formed along the direction of plotting with a vibration depth and period of 0.9 μm and 50 μm, respectively. The surface-modified 3D extrusion based scaffold exhibited enhanced mechanical property and improved initial cell attachment and proliferation compared to conventional extrusion based scaffolds.

2.6 Modification of scaffolds

2.6.1 Through combinational approach

Only a few number of additive manufacturing technologies has been used in clinical area, though these technologies are notably successful in overcoming the hurdle of desired complex scaffolds architecture. Therefore, still there is enough room to develop “smart scaffolds”, even from a scaffold design and manufacturing point of view. In this aspect combining different scaffolds fabrication techniques to fabricate scaffolds are gaining much interest [106]. As we know extrusion based additive manufactured
scaffolds have a poor cell seeding efficiency due to limitation in scaffold’s pore resolution. To overcome this problem a hybrid scaffold structure combining an extrusion based scaffolds fabrication technology with electrospinning has been proposed [107, 108]. The resulting hybrid structure consisting of macro sized strands and micro sized filaments which give mechanical support and enhance the cell entrapment efficiency of the hybrid scaffolds, respectively. Though the hybrid scaffolds resulted by combining the extrusion based scaffolds fabrication technology with electrospinning are very promising in the field of tissue engineering, more efforts are needed in the insulation of the additive manufacturing robot from the electrospinning high voltage collector [16]. Hence, hybrid scaffolds developed in our lab by combining conventional freeze drying technology with extrusion based scaffolds fabrication technology may be considered as a simple and effective manufacturing approach (described in chapter 6).

Surface topography like micro pores which play an important role for pheonotypic expression of osteoblast like cells [109], are difficult to obtain and control in the additive manufactured scaffold. This limitation can be addressed by integrating traditional porogen technique with the additive manufacturing technique [110]. To fabricate the desired scaffolds, at first, the mold was created by additive manufacturing and thereafter, the mold was filled with slurry material followed by drying and high temperature treatment. At high temperature the resign mold and porogen decomposes thermally leaving behind the desired scaffolds with interconnected macro pores and micro pores throughout the entire surface of the scaffolds.
2.6.2 Through surface modification

From the previous discussions it can be said that there has been significant research on the architecture, porosity and pore size of the additive manufactured scaffolds with pore dimensions and material properties being vital in promoting cell proliferation and de novo production of extracellular matrix. However, making scaffold surfaces more biocompatible and/or combining bioactive molecules are comparatively recent trends towards the development of next generation additive manufactured scaffolds for bone tissue engineering [52].

It is a common hypothesis that a bone like apatite layer bound to the surface of the scaffolds could be useful in bone tissue engineering. To provide an apatite coating through simulated body fluid (SBF) on the additive manufactured PCL scaffolds, the PCL scaffolds were treated with NaOH to provide carboxylate groups onto the surface and then dipped in aqueous CaCl$_2$ and K$_2$HPO$_4$.3H$_2$O alternately to deposit apatite nuclei on the surface. A dense and uniform apatite was formed on the surface modified scaffolds after incubation for 24 h in SBF [111]. Same type of apatite coating can also be formed on additive manufactured PCL scaffolds by introducing oxygen containing functional group on the scaffolds followed by creating nucleation site and after incubation for 24 h in SBF like the previous one [112]. Nucleation sites on the additive manufactured PCL scaffolds can also be created by impregnating scaffolds with a sodium silicate gel [113]. However, the dense coating on the surface of additive manufactured scaffolds failed to improve the osteoconductive properties of the scaffolds due to the flaking tendency of the apatite coating on the bars of the scaffolds. The flaking of the apatite coating happened due to the tensile force exerted by tissue sheets bridging adjacent struts of the additive manufactured scaffolds which ultimately resulted in poor
cell attachment, growth and mineralization [113]. In this context a thin biomimetic coating is desirable. Our study showed that a layer of thin composite coating of carbonated hydroxyapatite (CHA)-gelatin significantly improves the proliferative and osteoconductive properties of the additive manufactured PCL/TCP scaffolds [114]. In this study a CHA-gelatin composite coating with a thickness of 821 ± 53 nm was formed on additive manufactured PCL/TCP scaffolds through biomimetic co-precipitation process. Besides the apatite coating collagen mimetic peptide, GFOGER used as a coating on additive manufactured PCL scaffolds. GFOGER which is a synthetic triple helical peptide promotes osteoblastic differentiation via $\alpha_2\beta_1$ integrin receptor [115]. GFOGER coatings passively absorbed onto the PCL scaffolds, in the absence of exogenous cells or growth factors, significantly increased bone volume in non-healing femoral defects compared to uncoated scaffolds [116].

Additive manufactured scaffold based bone tissue engineering has only just started using growth factors (BMP, etc) or other biological molecules (HS, etc). BMP carriers play an important role in bone regeneration, for instance a non-compressible scaffold has been shown to promote better fusion in posterolateral spine than the clinically used collagen sponge [52]. Here, it should be noted that the clinically used collagen based carriers lack good mechanical properties. In this context, additive manufactured PCL/TCP scaffolds were pre-lyophilised with collagen-I to evaluate the *in vivo* delivery of rhBMP-2 in a critical sized rat cranial defect model [117]. The progression of healing in all modalities showed that the release of rhBMP-2 from additive manufactured PCL/TCP-collagen scaffolds is a clinically substantial approach to repair and regenerate critical size craniofacial bone defects. In another study rhBMP-2 delivered with immobilized heparin-gelatin modified additive manufactured poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV)/Ca-P scaffolds showed significantly enhanced alkaline
phosphatase activity and osteogenic differentiation markers compared with the rhBMP-2 loaded unmodified scaffolds [118]. As heparin sulphate polysaccharides improve the effects of growth factors on cell proliferation and differentiation, it can be hoped that heparin sulphate polysaccharides will intervene scaffold based bone tissue engineering significantly [52].

2.7 Conclusions

The need for bone TE scaffolds is socio-economic, and AM is showing great potential in this regard by providing patient specific scaffolds with controlled internal structure. Despite the advancements in AM scaffolds for bone TE, bone TE is still a long way off matching Nature’s ability to grow and repair tissues and organs. Hence, bone TE still has scope for enhancement. From this review it can easily be seen that scaffold technology is much more complex than illustrated by Figure 2.2. There are numerous elements or ways by which people are trying to improve the suitability of the scaffolds as can be seen in Figure 2.7. It can be hoped that in near future a true revolution will be perceived in the field of TE.

However, the related problems in this field are multidisciplinary; we need to act in multidisciplinary teams. There are lots of things that must be done before the availability of scaffold for clinical purpose. Though there are so many obstacles, there are good signs in the horizon. We should keep trying to mimic properties of the natural tissues like bone. We should receive the challenge to replicate what nature has given to us.
Figure 2.7 Elements contributing to scaffold technology.
Chapter 3

Screw Extrusion System (SES) and its Fabricated Scaffolds

3.1 Introduction

Screw extrusion system (SES) is a low-cost additive manufacturing technology enabling the fabrication of design dependent complex 3D scaffolds using various types of raw materials with high reproducibility [11, 12, 92, 96]. Similar to conventional polymer extrusion processes SES uses temperature to control the material state. The main features of this SES are as follows:

- Material feeding: small volume can be fed, making it suitable for expensive materials.
- Material liquification using a controlled heating element.
- Material movement through the barrel and nozzle due to screw assisted pressure.
- Material extrusion in filament form with a controlled manner to a predefined path.
- Extruded filament bonding to the previous layer to form design dependent 3D scaffolds.

The parameters which would determine the quality of the fabricated scaffolds include the motor speed, dispensing speed, the melt temperature, the platform temperature, layer thickness, nozzle size and road width [92, 95]. Understanding different process parameters relating to the scaffolds fabrication process is important for coherent scaffold fabrication.
In this chapter some important features of in-house SES will be discussed first, and after that PCL/TCP scaffolds fabricated via this in-house SES will be evaluated in terms of process parameters, mechanical and biological properties.

### 3.2 Main features of in-house SES

The in-house SES consists of a robotic-arm, extruder part, and heater similar to conventional FDM machine. Figure 3.1 shows the photograph of the in-house extruder part. The extruder part mainly consists of screw, extruder body part and nozzle which are briefly discussed in the following sections.

![In house extruder part](image)

**Figure 3.1 In house extruder part.**

#### 3.2.1 Extruder screw

Feed screws are used in the extrusion process, which convey plastic pellets through a heated barrel where they are melted and forced out of a die. Other functions of the screw include mixing material together and generating pressure so that material can be forced through the die. Screws are attached to a motor through a bearing and rotated in
the barrel of a machine. Plastic is conveyed by the flights on the screw. Flights are the angled disks that are on the shaft of the screw. Typical clearance between the flights and the barrel are about 0.025 mm [119]. Flight depth is the distance from the tip of the flight and shaft of the screw. Flight depth varies between screw sections.

Extruder screws have three sections (Figure 3.2): feed section, transition section, and metering section. The feed section has the greatest flight depth and is used to convey un-melted pellets. The transition section has a decreasing flight depth which helps to melt plastic and build pressure. Plastic is completely melted in this section. The metering section has the lowest flight depth and creates a high amount of shear which causes a pressure build up.

![Extruder screw diagram](image)

Figure 3.2 Extruder screw. Adapted from [119].

### 3.2.2 Extruder body part

Figure 3.3 shows different parts of the extruder frame. The extruder main back support and the front screw supports are both 27 mm from the centre of the extruder screw (Figure 3.4a). Thus this is a screw centric design in which the supports are built around the screw to allow equal loading between the front and back supports. Equal loading will prevent high stress generation upon the main back frame and thereby may have effect on reducing the upward thrust generated by the rotating screw. However, to
reduce the upward thrust by the rotating extruder screw, front screw supports has been incorporated as shown in figure 3.4b.

Figure 3.3 Parts of extruder frame.

Figure 3.4 (a) Extruder centric frame and (b) Front support screws.
Chapter 3.

There are two functions to the extruder front screw supports. When extrusion is in progress, the screw experiences an upward thrust that is handled by the frame. The two frontal supports take some of the load that the rear back frame experiences, distributing the stress induced upon the frame. As the frame is made up of aluminium, a relatively soft material, distributing the stress would be beneficial in preserving the frame structure. Secondly, the frontal screw supports can pull the top screw support platform and barrel support platform to prevent the backward bending of the main back frame during extrusion. By doing so, the screw will not tilt and scratch the barrel when operating at high rotational speed.

Figure 3.5 Vertical barrel replacement.

In the extruder frame the barrel is attached vertically to the barrel support by screws as shown in Figure 3.5. Therefore, during cleaning the screw can be accessed easily just by unscrewing the six screws attaching the barrel to the barrel support, and barrel remains untouched which eventually prevents the misalignment that can happen if barrel support need to be removed repeatedly with barrel during screw cleaning.
Figure 3.6 Hopper design. (a) Slope cut into piece that acts as hopper and (b) Disk shaped hole cut into platform for barrel attachment and six smaller circles on perimeter representing placement of M5 screws.

However, in order to use the screws to attach the barrel and barrel support a platform which is thick enough is needed. Hence a thick platform is designed with six M5 screw holes drilled into it to allow the attachment of the screws from the barrel at the bottom of the platform. In the top part of the platform a slope cut has been introduced so that this can also act as a hopper. Thus the hopper and barrel support is integrated into a single piece as demonstrated by Figure 3.6.

### 3.2.3 Extruder nozzle

Figure 3.7 illustrates the 0.4 mm diameter nozzle design of our in-house screw extrusion system. There is a slope towards the exit tip of the nozzle, which is to ensure uniform loading from the fluid pressure along the nozzle wall.

Figure 3.7 Nozzle of the in-house SES.
3.3 Evaluation of scaffolds fabricated via in-house SES

3.3.1 Scaffold Material

Poly($\varepsilon$-caprolactone) ($M_n$: 80,000) and tri-calcium phosphate (TCP) were purchased from Sigma-Aldrich (Singapore) and Progentix (The Netherlands), respectively. PCL/TCP composite with 20 wt\% of TCP was prepared by blending TCP powder into PCL using Brabender Mixer at 80 °C.

3.3.2 Scaffold Design

Once the material is poured into the screw extrusion system, the material becomes fused to a semi liquid state. The fused material is then extruded through the nozzle due to pressure created by screw rotation. The extruded material is then deposited in layers onto a flat base. The material solidifies and bonds to the preceding layer. Scaffolds are fabricated in layers, where a layer is built by extruding a small bead of material, or road, in a particular lay down pattern, such that the layer is covered with the adjacent filaments called roads. After a layer is completed, the height of the extrusion head is increased and the subsequent layers are built to construct the entire scaffold. Some structural features of the scaffold are shown in the Figure 3.8. RW is defined as the diameter of the circular cross-section of laid filament, ST is the vertical distance between the filament centres of adjacent layers, FG is the edge-to-edge horizontal distance between adjacent filaments, FD is the centre-to-centre horizontal distance between adjacent filaments and LG is defined as the edge-to-edge vertical distance between layers of the same filament alignment.
3.3.3 Scaffold fabrication

PCL/TCP scaffolds were fabricated with a laydown pattern of 0º/90º and filament distance of 1.5 mm. The processing temperature was 85 ºC and the size of the scaffolds fabricated were 30 mm × 30 mm × 7 mm.

The effect of dispensing speed on porosity and modulus were studied by varying the dispensing speed, i.e. the speed by which robot arm is moving or the speed at which material is laying down. In this experiment we also investigated the effect of different nozzle size on properties of scaffolds by using two nozzles having the diameter of 0.4 mm and 0.5 mm. The rotational speed of the screw was kept constant at 25 rpm and dispensing speed was varied in a considerable range. After making all the scaffolds, the scaffold blocks were removed from the base and the edges were trimmed off by sharp blade to get uniform edged bulk scaffolds of size 30 mm × 30 mm × 7 mm (Figure 3.9). For testing these scaffolds were cut to the size of 5 mm × 5 mm × 7 mm.
3.3.4 Characterization of the scaffold

3.3.4.1 Porosity measurement

The porosity of the scaffolds was determined theoretically and experimentally. The theoretical value was measured from the SEM measurements by using the formula as described below [28, 120],

\[
\text{Porosity, } P = 1 - \frac{V_{\text{scaffold}}}{V_{\text{cube}}} = 1 - \left[ \frac{\pi}{4} \times \frac{1}{d_2/d_1} \times \frac{1}{d_3/d_1} \right]
\]

where \( P \) is the scaffold porosity, \( d_1 \) the fiber diameter, \( d_2 \) the fiber spacing, and \( d_3 \) the layer thickness. \( V_{\text{scaffold}} \) is the volume of the porous scaffolds, whereas \( V_{\text{cube}} \) is the volume of the cube having similar dimension of scaffolds. The values of \( d_1 \), \( d_2 \) and \( d_3 \) have measured from the SEM images.

Figure 3.9 Bulk scaffold block with uniform edge after trimming off.

Figure 3.10 Schematic of (a) porosity calculation and (b) strand layout for 0/90° pattern.
It should be mentioned here that, ideally $d_1$ should be equal to $d_3$. However, to enhance fiber adhesion between two layers and to compensate for gravitational effects on the circularity of the extrusion, we have reduced layer thickness approximately 8% with respect to the nozzle diameter. The reduction in layer thickness by 8% has chosen based on few experimental trials.

The experimental porosity value of the scaffold was measured gravimetrically as reported previously [121]. In this technique, porosity is defined by the following formula:

$$\text{Porosity}, P = \frac{V_{\text{pore}}}{V_{\text{pore}} + V_{\text{polymer}}}$$

where, $V_{\text{pore}}$ is the part of the volume occupied by pores and $V_{\text{polymer}}$ is the volume occupied by the polymer. Let $m'_s$ be the mass of the scaffold with trapped water and $m''_s$ the mass of the dry scaffold. The mass of water located in the pores, $m'_{w_\text{pores}} = m'_s - m''_s$. Taking into account the density of water $\rho_w$, the amount of water located in pores gives their volume,

$$V_{\text{pore}} = \frac{m'_s - m''_s}{\rho_w}.$$

However, the volume of the scaffold taken by the polymer can be obtained by measuring the density of the corresponding bulk material $\rho_b$

$$V_{\text{polymer}} = \frac{m''_s}{\rho_b}.$$
\( \rho_b \) was determined to be 1.2627 \( \text{gm/cm}^3 \) by weighing the a block of PCL/TCP composite with no pores in water and air. A balance with 0.1 mg sensitivity was used for the weighing purposes.

3.3.4.2 Morphology

- Scanning Electron Microscopy (SEM)

Scaffold morphology and pore size was studied using SEM. Scaffolds were gold sputtered by using a JEOL fine sputter coater (JFC-1200) for 15 seconds at 10 \( \mu \text{m} \). The SME images were taken by Philips XL30 FEG and using the beam intensity of 15 kV and the gaseous secondary electron detectors at 1.3 Torr.

3.3.4.3 Mechanical experiments

Compression tests were conducted on scaffolds by using an Instron 4502 Uniaxial testing system and 5 kN load cell (Canton, MA, USA). For each group 4 samples were tested. The scaffolds were compressed in the Z-direction at a rate of 1 mm/min up to a strain level of approximately 80%. By strain level of 80%, it is meant that the scaffolds were compressed up to their 80% of the height. The stress-strain (\( \sigma-\varepsilon \)) curves were obtained to evaluate the compressive stiffness (modulus). The stiffness was calculated from the stress-strain curve as the slope of the initial linear portion of the curve, with any toe region from the initial settling of the specimen neglected.
3.3.5 *In-vitro cell culture study*

3.3.5.1 Cell seeding on scaffolds

Tissue culture media Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin (pen-strep) were purchased from Gibco. Trypsin-EDTA was purchased from Thermo Scientific Hyclone.

Cryopreserved porcine BMSCs (passage 2), previously isolated from the iliac crest of pigs, were plated and expanded in T75 tissue culture flasks in DMEM supplemented with 10% FBS and 1% antibiotics until getting sufficient cells (not beyond passage 4). Medium was changed twice per week and cells were detached by trypsin-EDTA and passaged into fresh culture flasks at a ratio of 1:3 upon reaching confluence. Cultures were incubated at 37 °C in a humidified atmosphere containing 95% air and 5% CO₂.

First the scaffolds were sterilized with 70% ethanol for 30 min and then further sterilized under UV light for 30 minutes followed by drying in sterile condition at room temperature for 2 hours. Approximately $1.0 \times 10^5$ BMSCs were seeded onto each process optimized PCL/TCP scaffolds. The cell-scaffold constructs were cultured in 24-well tissue culture plates for up to 21 days in growth medium, and at specific time points cell-scaffold constructs were assessed and observed for proliferation.

3.3.5.2 Morphology of the cell-scaffolds constructs

Confocal laser microscopy and SEM were used to assess cell viability, morphology and attachment in vitro. Cells were stained with 2 μg/ml of fluorescein diacetate (FDA) (1 mg/mL, Molecular probes) to stain the cytoplasm of the live cells with green fluorescence. During FDA staining, samples were incubated at 37 °C for 15 min and followed by rinsing with phosphate buffer saline (PBS). Cells were then counterstained
with propidium iodide (PI) 5 μg/mL (1 mg/mL, Molecular probes) to stain the nucleus of the dead cells with red fluorescence. During PI staining the samples were kept at room temperature for 2 min and then rinsed with PBS. These samples were mounted for viewing under confocal laser microscope (IX 70, Olympus). Depth projection images were constructed from up to 30 horizontal image sections (12 μm each) through the stained cell-scaffold constructs using FV1000 Viewer (Ver.1.7a) software. After confocal imaging, cell-scaffolds constructs were fixed in 10% formalin overnight and then dehydrated through a series of graded ethanol solutions (5, 10, 20, 40, 60, 80, 90 and 100%), each for 10 min for dehydration. Finally, the samples were dried overnight at room temperature. Dried cellular constructs were gold sputtered and observed under SEM at an accelerating voltage of 10 KV.

3.3.5.3 AlamarBlue

The proliferation of BMSCs was analysed with metabolic assay AlamarBlue which is a simple, rapid, reliable and cost-effective measurement. AlamarBlue detects cell viability by utilizing a noncytotoxic reduction–oxidation (redox) indicator and hence non-destructive [122]. First culture medium was replaced with DMEM containing 5% (v/v) AlamarBlue (BioSource International, Camarillo, CA) and was incubated. After 2 hours of incubation, a microplate reader (GENios; Tecan Group, Maennedorf, Switzerland) was used to measure the absorbance of 100 μL samples of each well at wavelengths of 560 and 595 nm after shaking the well plate for 8 seconds. The data were normalized with 100% media and 100% AlamarBlue readings.
3.3.6 Results and discussion

3.3.6.1 Porosity and dispensing speed

The term dispensing speed was defined by relative movement between the nozzle and the table. The range of dispensing speed was 6 mm/s – 12 mm/s for 0.4 mm nozzle diameter and for 0.5 mm nozzle diameter it was 10 mm/s – 16 mm/s while all other parameters were kept constant. Porosity of the scaffolds was calculated from SEM image measurement and also by gravimetrically by swelling the sample in water. Table 3.1 summarised the result of theoretical porosity measured from the SEM images. The SEM images of the scaffolds for different dispensing speeds have been given in Figure 3.11 and Figure 3.12 for 0.4 mm nozzle diameter and in Figure 3.13 and 3.14 for 0.5 mm nozzle diameter. The spacing between fibers (d2) is assumed constant throughout the whole scaffolds.

<table>
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<tr>
<th>Dispensing speed (mm/s)</th>
<th>d1 (mm)</th>
<th>d2 (mm)</th>
<th>d3 (mm)</th>
<th>Porosity (%)</th>
</tr>
</thead>
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<tr>
<td>Nozzle diameter 0.4 mm:</td>
<td></td>
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<tr>
<td>6</td>
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<td>0.52 ± 0.07</td>
<td>1.5</td>
<td>0.57 ± 0.05</td>
<td>75</td>
</tr>
<tr>
<td>16</td>
<td>0.42 ± 0.04</td>
<td>1.5</td>
<td>0.41 ± 0.03</td>
<td>77</td>
</tr>
</tbody>
</table>
Figure 3.11 SEM images of scaffolds fabricated by 0.4 mm nozzle diameter with different dispensing speed.

Figure 3.12 SEM images of some irregular dispensing at higher (12 mm/s) dispensing speed for 0.4 mm nozzle diameter.

Figure 3.13 SEM images of scaffolds fabricated by 0.5 mm nozzle diameter with different dispensing speed.

At higher speed the extruded material is not getting enough time to be positioned down. The net result is a pulling effect.

Figure 3.14 SEM images of some irregular dispensing at higher (18 mm/s) dispensing speed for 0.5 mm nozzle diameter.
The relation between theoretical and experimental porosity vs. dispensing speed for different nozzle diameter is illustrated in Figure 3.15. From this graph it can be seen that for 0.4 mm nozzle diameter the experimental porosity varies from 63.88% to 73.77%. The porosity of 0.4 mm nozzle diameter at dispensing speed 8 mm/s closely matches the porosity of 0.5 mm nozzle diameter at dispensing speed 14 mm/s. Almost 1.7 times higher speed is required to get the porosity from 0.4 mm nozzle diameter compared to the 0.5 mm nozzle diameter. At higher dispensing speed the extruded filaments do not get enough time to settle down and thus the laying down of filaments becomes irregular due to the pulling phenomenon as illustrated in Figure 3.12 and Figure 3.14 for 0.4 mm and 0.5 mm nozzle diameter, respectively. As noticed from Figure 3.11 and Figure 3.13 a 0.4 mm nozzle diameter 10 mm/s dispensing speed works well, whereas, for 0.5 mm nozzle diameter 16 mm/s dispensing speed works well.

Figure 3.15 Theoretical and experimental porosity vs. dispensing speed for different nozzle diameter.
3.3.6.2 Modulus and dispensing speed

The modulus of the scaffold starts decreasing with the increase of the dispensing speed. This is because with the increase in the dispensing speed the diameter of the extruded material starts decreasing due to pulling effect. Figure 3.16 shows the relationship between modulus and dispensing for different nozzle diameter.

Figure 3.17 shows the relationship between compressive modulus and porosity. For 68.89% porosity in 0.5 mm nozzle diameter the modulus is 42.12 MPa. Whereas, for 69.52% porosity in 0.4 mm nozzle diameter results in 7.43 MPa. For similar porosity the modulus is 5.5 times higher in 0.5 mm nozzle diameter. It may be because; the diameter of the filament of 0.5 mm nozzle diameter at that parameter was 0.519 mm. However, the filament diameter for 0.4 mm nozzle diameter at that point was 0.413 mm. Another important thing is, at the higher porosity the modulus drops in a rapid manner. This may be because at higher porosity value the structure becomes too porous and collapses suddenly.

From the process optimization experiments PCL/TCP scaffolds fabricated with 0.4 mm nozzle diameter and dispensing speed of 10 mm/s have been chosen for further investigation. *In vitro* evaluation of this process optimized PCL/TCP scaffolds has been discussed in the next section.
Figure 3.16 Modulus vs. dispensing speed for different nozzle diameter.

Figure 3.17 Compressive modulus vs. porosity for different nozzle diameter.
3.3.6.3 *In vitro cell culture results*

Figure 3.18 shows the SEM images of BMSCs cultured on PCL/TCP scaffolds at day 7 and day 21. At day 7, cells have attached and started to spread on PCL/TCP scaffolds. At day 21, cells spread well onto the PCL/TCP scaffolds and cell-sheet accumulation were also noticed.

![SEM images of BMSCs cultured on PCL/TCP scaffolds at day 7 and day 21.](image)

**Figure 3.18** SEM images of cell-scaffold construct of PCL/TCP scaffolds at (a) day 7 and (b) day 21.

Figure 3.19 shows the confocal laser microscopy image of BMSCs cultured on PCL/TCP scaffolds at different time points. BMSCs showed a steady growth with respect to time on SES fabricated PCL/TCP scaffolds.

![Confocal laser microscopy of cells within the scaffolds of PCL/TCP.](image)

**Figure 3.19** Confocal laser microscopy of cells within the scaffolds of PCL/TCP at (a) day 7, (b) day 14 and (c) day 21.
AB results show a steady growth in metabolic activity of BMSCs cultured on SES fabricated PCL/TCP scaffolds [Figure 3.20]. All the SEM, confocal and AB results demonstrated that SES fabricated PCL/TCP scaffolds are biocompatible.

![Fluorescence normalized with medium and AB over time](image)

Figure 3.20 BMSCs metabolism analysis using AlamarBlue.

### 3.3.7 Conclusions

The in-house SES was designed and its various process parameters were investigated successfully to fabricate 3D porous PCL/TCP scaffolds. The process optimized PCL/TCP scaffolds showed promising results in biological evaluation. The preliminary results not only demonstrates the feasibility of using the in-house SES to fabricate PCL/TCP scaffolds for bone tissue engineering but also encouraging enough to initiate further study to improve the mechanical and functional performance of the in-house SES fabricated PCL/TCP scaffolds. In the next chapter the way to improve mechanical properties of PCL/TCP scaffolds will be discussed.
Chapter 4

Improvement of Mechanical Properties of Additive Manufactured PCL/TCP scaffolds

4.1 Introduction

Despite the high regenerative capacity of bone, large bone defects due to high energy trauma, bone tumour resections, congenital malfunction and severe non-union fractures require a bone substitute for regeneration, which is about 10% of all orthopaedic operations worldwide [2]. Common clinical bone substitutes include xenograft, allograft and autogenous cancellous bone graft from the ilium, chest or tibial tuberosity. Autogenous cancellous bone graft is the most common bone substitute because of its biological properties and lack of possibility of disease transmission or host rejection [3]. Nevertheless, its use is severely hampered by its short supply, temporary disruption of donor site bone structure and considerable donor site morbidity associated with the harvest [3, 4]. Therefore, the development of new synthetic bone substitute or scaffold that could be used instead of autogenous cancellous bone graft, has become a key research topic in bone tissue engineering [5-7, 123].

An ideal scaffold aims to mimic the mechanical and biochemical properties of the native tissue. Therefore, one fundamental hypothesis is to design scaffold to provide a biomimetic mechanical environment to withstand in vivo stress and loading [13]. Specifically, it was proposed that the implanted scaffold should match the stiffness and strength of native tissue [6, 7], which ranges from 50 to 500 MPa and 3 to 12 MPa for cancellous bone, respectively [15, 124].
Additive manufacturing (AM) technology is a computer controlled fabrication process and holds the key for generic solution in automating scaffolds production because of its consistency and high reproducibility. It has great potential over conventional manual-based fabrication techniques, as the scaffolds fabricated by AM are design dependent, whereas the scaffolds fabricated by conventional techniques is highly process dependent [8]. Poly (ε-caprolactone) (PCL) is a bioresorbable and thermoplastic aliphatic polyester, and has been used preferably to fabricate scaffolds with AM technology because of its easiness in formability at relatively low temperature and good melt viscosity [49, 51, 125, 126]. In order to increase the mechanical properties and bioactivity of these PCL scaffolds, pristine ceramic particles such as hydroxyapatite (HA) and tricalcium phosphate (TCP) have been mixed into the PCL matrix directly [12, 14, 127, 128].

Nevertheless, to date, only a slight improvement in compressive modulus and strength was achieved with PCL/ceramic composite scaffolds compared with those of PCL scaffolds. As an example, PCL/ tricalcium phosphate (TCP) composite scaffolds exhibited compressive modulus of 9.3 MPa and compressive strength of 3.1 MPa, while PCL scaffolds exhibited compressive modulus of 2.7 MPa and compressive strength of 1.5 Mpa [14]. These PCL/TCP scaffolds are remarkably weak for cancellous bone tissue engineering. As reported recently in the review paper by Rezwan K. et al., the limited improvement on mechanical properties of polymer/ceramic composite scaffolds is because of the relatively low interfacial bonding between ceramic filler and polymer matrix [15]. Another study has also demonstrated that effective stress transfer across interface between polymer and ceramic filler is crucial for producing composite scaffolds with adequate mechanical properties [129].
Here, high performance additive manufactured PCL/TCP scaffolds have been developed for bone tissue engineering by using different binders. In part I and part II of this chapter silanized PCL/TCP and POSS modified PCL/TCP composite has been described in details, respectively. To synthesize silanized PCL/TCP composite TCP was modified using 3-glycidoxypropyl trimethoxysilane (GPTMS) to enhance the interfacial bonding between TCP and PCL matrix. To ensure the formation of covalent bonding between TCP(Si) and PCL, the dried PCL/TCP(Si) composites was annealed at 120 °C for 2 hr. To synthesize POSS modified PCL/TCP composite PCL, TCP and POSS were melt compounded at 120 °C for 20 min. The dried POSS modified PCL/TCP (PCL/TCP(POSS)) composite was further modified through annealing at 180 °C for 2 hr. After synthesizing both of the composites, scaffolds were fabricated using an in-house screw extrusion system (SES). The fabricated scaffolds were evaluated in terms of physical, mechanical and biological characterizations. We hypothesized that such developed scaffolds would provide suitable mechanical properties for using in bone tissue engineering applications without any detrimental effect of binders on biocompatibility.
4.2 Part I – Silanized PCL/TCP(Si): composite synthesis, scaffolds fabrication and characterization

4.2.1 Materials and methods

4.2.1.1 Materials

Poly(ε-caprolactone) (PCL) ($M_n$: 80,000) and 3-glycidoxypropyl trimethoxysilane (GPTMS) were purchased from Sigma-Aldrich, Singapore. Tri-calcium phosphate (TCP) was purchased from Progentix, The Netherlands.

4.2.1.2 Synthesis of PCL/TCP(Si)

Surface activation of TCP was carried out by stirring TCP in 2% phosphoric acid (1:2 w/w) at room temperature for 2 hr followed by sonicating for 5 minutes [130]. The surface activated TCP was washed first with distilled water for 3 times to remove free phosphoric acid and then with acetone for 3 times to remove water, and suspended in acetone. GPTMS was then added into TCP acetone suspension and refluxed at 75 °C for 24 hours. GPTMS modified TCP (TCP(Si)) was collected through filtration followed by thorough wash with acetone to remove free GPTMS. The TCP(Si) was incorporated into PCL through homogenizing PCL/TCP(Si) acetone solution at 8000 rpm for 15 minutes. After evaporating off acetone, the homogenized composite was vacuum dried at 50 °C for 1 week. Finally, PCL/TCP(Si) composite was melt compounded at 120 °C for 20 minutes and annealed at 120 °C for 2 hours.

4.2.1.3 Fabrication of the PCL/TCP(Si) scaffolds by SES

Additive manufactured PCL/TCP(Si) composite scaffolds were fabricated using an in-house screw extrusion system (SES) with screw rotational speed of 25 rpm and nozzle
diameter of 0.4 mm at processing temperature of 85 °C. The center to center distance of the scaffold struts was set to 1.5 mm. Each layer of the scaffolds was fabricated with the designed pattern of 0°/60°/120° orientation. The size of the scaffolds used for mechanical test was 5 mm × 5 mm × 8 mm and that for cell culture was 5 mm × 5 mm × 3.5 mm. PCL/TCP scaffolds were also fabricated as control.

4.2.1.4 Characterization

The surface chemical structure of TCP(Si) was characterized by XPS (Thetaprobe from Thermo Scientific), using a source of monochromated Al K$_\alpha$ (1486.6 eV) along with flood gun for surface charge compensation (energy 2 eV, emission current 100 mA). Detector was set at standard lens mode CAE (contact analyzer energy) and passed energy for survey spectra is 200 eV and 40 eV for high resolution spectra. Data analysis was done using the Avantage software. FTIR spectra of TCP and TCP(Si) minced into the KBr pallet were recorded on a Bio-Rad 165 FT-IR spectrophotometer; 64 scans were signal-averaged with a resolution of 2 cm$^{-1}$ at room temperature. Differential scanning calorimetry (DSC) measurements were performed using a TA Instruments 2920 differential scanning calorimeter equipped with an auto-cooling accessory and calibrated using indium. The following protocol was used for each sample: heating from room temperature to 120 °C at 20 °C min$^{-1}$, holding at 120 °C for 2 minutes, cooling from 120 to -30 °C at 5 °C min$^{-1}$, and finally reheating from -30 to 170 °C at 5 °C min$^{-1}$. Data were collected during the second heating run. The degree of crystallinity was calculated according to the following equation:

Crystallinity (%) = (Δ$H_m$/ 0.8/Δ$H_m^o$) × 100%; where $H_m$ is the melting enthalpy (J/g) of composite; 0.8 is the weight percentage of PCL in the composite and Δ$H_m^o$ (139.5 J/g) is the melting enthalpy of 100% crystalline PCL [131].
Compressive modulus and compressive strength of the scaffolds were measured using an Instron 4502 uniaxial testing system with 5 kN load cell at a rate of 1 mm/minute. All scaffolds had the size of 5 mm × 5 mm × 8 mm and 0º/60º/120º lay down patterns. Ten scaffolds were tested from each group and measurements were reported as mean ± standard deviation.

The surface morphology of the scaffolds was studied with a Quanta 200F field emission scanning electron microscope (FESEM) at a beam intensity of 10 kV after gold sputtering using a JFC-1200 fine sputter coater.

4.2.1.5 Cell seeding on scaffolds

BMSCs (1.8×10^5 cells) were added onto each of the PCL/TCP and PCL/TCP(Si) scaffold according to the protocol described in the in vitro cell culture study section of chapter 3. The cell-scaffold constructs were cultured up to 10 days in growth medium, and at specific time points cell-scaffold constructs were assessed and observed for proliferation. For osteogenic induction, after 10 days of culture in growth medium, an osteogenic cocktail of dexamethasone (10 nM), ascorbic acid (50 μM) and β-glycerolphosphate (10 mM) (Sigma) was added. The cell-scaffold constructs were cultured further for 28 days post induction and samples were assessed at definite time points for gene and protein expressions.

4.2.1.6 Morphology of the cell-scaffold constructs

SEM and confocal laser microscopy were used to assess cell morphology, viability and attachment in vitro according to the protocol described in the in vitro cell culture study section of chapter 3.
4.2.1.7 PicoGreen® Assay

Proliferation of BMSCs in cultured cell-scaffold constructs were studied using PicoGreen® assay as per manufacturer’s protocol (Molecular Probes, PicoGreen dsDNA Quantitation kit). Total DNA from cells in the constructs were extracted via freeze-thaw cycles. DNA amount in the cell lysate were then assayed by mixing with DNA binding PicoGreen® dye followed by spectrophotometer quantisation.

4.2.1.8 Gene Expression (Real-time RT-PCR)

At specified time points, the cell-scaffold constructs were lysed with Trizol (Invitrogen) and thoroughly vortexed. The samples in Trizol were stored at -80 °C until RNA isolation. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) with Rnase-Free DNase (Qiagen) according to the manufacturer’s protocol.

Reverse transcription was performed with the QuantiTect Reverse Transcriptase kit (Qiagen) to make the required cDNA. Quantitative PCR was performed for the quantification of gene expressions using the primers listed in Table 4.1 and QuantiTect SYBR Green PCR kit (Qiagen) Mx3000P Real-Time PCR System (Stratagene). Target genes were normalized against the GAPDH expression.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GCTTTGCCCGCGATCTAATGTTTC</td>
<td>GCCAAATCCGTTCACTCGACCTT</td>
</tr>
<tr>
<td>CBFA1</td>
<td>GAGGAACCGTTTCACTTTACTG</td>
<td>CGTTAACCACCGGCA CGA G</td>
</tr>
<tr>
<td>COL1</td>
<td>CCAAGAGGGAGGGCGCAAGAGAAGG</td>
<td>GGGGCAGACGCGGCGACACTC</td>
</tr>
<tr>
<td>OCN</td>
<td>TCAACCCCGACT GCGACGAG</td>
<td>TTGGACGAGCTG GGATGATGG</td>
</tr>
</tbody>
</table>

4.2.1.9 Western Blot Study (WB)

Protein extracts were harvested from the cell-scaffold constructs with ice-cold radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific). Protein lysates were
purified and concentrated using NanoSep columns (Pall 3K). Total protein quantity was quantified using microBCA assay (Pierce) as per manufacture’s protocol. Proteins were denatured at 90 °C for 5 minutes, resolved by 10% SDS-PAGE (polyacrylamide gel electrophoresis) and transferred to nitrocellulose membranes (Bio-Rad). After blocking with 1% non-fat milk in tris-buffered saline (TBS) for 1 hour, the membranes were then incubated for 1 hour with primary antibodies: polyclonal rabbit-anti-human beta-actin (Delta Biolabs), osteonectin (ON), osteocalcin (OCN) (Santa Cruz) or osteopontin (OPN) (Abcam). The primary antibodies were diluted from 1:500 to 1:2000 in TBS with 0.1% Tween (TBST). After three washes with TBST, membranes were next incubated with rabbit anti-goat HRP-conjugated secondary IgG (Zymed) at 1:15,000 for 1 hour, followed by another three washes with TBST. Immunoreactive bands were visualised and detected using the SuperSignal Chemiluminescent reagent (Pierce) on the VersaDoc Imaging Systems (Bio-Rad). The intensities of the bands were quantitatively analyzed using densitometry (Quantity-One, Bio-Rad). Target proteins were normalized against the β-actin expression.

4.2.1.10 Statistical Analysis

All the data presented are expressed as mean ± standard deviation. An unpaired student’s t-test was used to test the significance level of the data. Differences were considered statistically significant at p<0.05.
4.2.2 Results and Discussions

4.2.2.1 PCL/TCP(Si) composite preparation and scaffolds fabrication

In order to prepare additive manufactured PCL/TCP scaffolds with adequate mechanical property for cancellous bone tissue engineering, the interfacial bonding between PCL and TCP was enhanced by modifying TCP using GPTMS as a coupling agent. The main idea is that the epoxide group from GPTMS attached to TCP could react with the end groups of PCL chain (-OH and -COOH) to form covalent bond during melt-compounding and the followed annealing. Figure 4.1 shows the schematic diagram of TCP modification as well as the covalent bond formation between TCP(Si) and PCL. First, hydroxyl group was introduced onto TCP by stirring its suspension in a diluted phosphoric acid (2 wt%) for 2 hours as reported previously [130]. Then, GPTMS was covalently bonded onto the surface of TCP via condensation reaction with the introduced hydroxyl group on TCP. The successful modification of TCP was verified by X-ray photoelectron spectroscopy (XPS) and Fourier transform infrared spectroscopy (FTIR). As shown in Figure 4.2, while only calcium, phosphorous and oxygen are detected on the surface of non-treated TCP using XPS, additional silicon peak (Si(2p): 99.8 eV; and Si(2s): 149.7 eV) from GPTMS is observed in the XPS spectrum of TCP(Si), confirming the presence of GPTMS on TCP. In the FTIR spectrum of TCP(Si) (Figure 4.3b), observation of additional characteristic epoxide band at 873 cm\(^{-1}\) in comparison to that of TCP also confirms the presence of GPTMS on TCP(Si). TCP(Si) with varying concentration of GPTMS was synthesized to study the influence of GPTMS content on the mechanical property of PCL/TCP(Si) scaffolds.

The synthesized TCP(Si) was suspended in acetone and mixed into PCL acetone solution by homogenizing at high speed. After evaporating off acetone using rotary
evaporator, the PCL/TCP(Si) composite was melt-compounded at 120 °C for 20 minutes followed by annealing at 120 °C for 2 hours to facilitate the formation of covalent bond between epoxide from TCP(Si) and –OH or –COOH from PCL. Similar chemical reaction has been reported previously [132, 133].

(A) TCP modification:

\[
\text{TCP} \quad \rightarrow \quad \text{TCP} + \text{PTMS} \quad \rightarrow \quad \text{TCP} + \text{CH}_{2} - \text{CH}_{3}
\]

\[
\text{GPTMS:} \quad \text{CH}_{2}O - \text{Si} - \text{O} - \text{O} - \text{O} - \text{CH}_{3}
\]

(B) Reaction between epoxide and terminal groups of PCL:

\[
\text{TCP} + \text{CH}_{2} - \text{CH}_{3} \quad + \quad \text{HO} - \text{C} - \text{PCL} \quad \xrightarrow{\text{Compounding}} \quad \text{TCP} + \text{OH} - \text{C} - \text{PCL}
\]

\[
\text{TCP} + \text{CH}_{2} - \text{CH}_{3} \quad + \quad \text{HO} - \text{PCL} \quad \xrightarrow{\text{Compounding}} \quad \text{TCP} + \text{OH} - \text{PCL}
\]

Figure 4.1 Schematic diagram of TCP modification.
Figure 4.2 XPS spectra of (a) TCP and (b) TCP(Si).

Figure 4.3 FTIR spectra of (a) TCP and (b) TCP(Si).
Additive manufactured PCL/TCP(Si) scaffolds (Figure 4.4) were fabricated from the above synthesized PCL/TCP(Si) composite using an in-house SES. SES is a potential melt extrusion based AM technology and has been used to fabricate scaffolds with specifically tailored pore geometry and architectural pattern [12]. In this study, the fabricated scaffolds were 100% interconnected with pore size of 750-950 μm and overall porosity of 65%. Additive manufactured PCL/TCP scaffolds with the same dimension were also fabricated as control.

![Image of SES fabricated scaffolds.](image)

Figure 4.4 Overview of the SES fabricated scaffolds.

The compressive modulus and strength of PCL/TCP(Si) scaffolds with varying content of GPTMS were measured by compression testing, and compared with those of PCL/TCP scaffold. As shown in Figure 4.5, both compressive modulus and strength of PCL/TCP(Si) scaffolds are significantly higher than those of PCL/TCP scaffold, and PCL/TCP(Si) scaffold with 10 wt% of GPTMS referred to TCP exhibits the maxima compressive modulus of 82.6 MPa and compressive strength of 4.0 MPa, which are 6.0 and 2.3 times of those of PCL/TCP scaffolds, respectively. It should be noted that both the compressive modulus and strength of PCL/TCP(Si) scaffolds with GPTMS content higher than 5 wt% fall in the range of mechanical properties of cancellous bone (50 to 500 MPa and 3 to 12 MPa), whereas PCL/TCP scaffold are remarkably weaker. As PCL/TCP(Si) scaffold with 10 wt% of GPTMS referred to TCP exhibits the best
mechanical properties, it has been chosen for *in vitro* evaluation, and denoted as PCL/TCP(Si).

Figure 4.5 Compressive modulus (a) and compressive strength (b) of PCL/TCP and PCL/TCP(Si) scaffolds with various content of GPTMS referred to TCP.

PCL is a semi-crystalline polymer, and the crystallinity degree of PCL in PCL/TCP and PCL/TCP(Si) with various GPTMS content were measured using differential
scanning calorimeter (DSC) and the results were listed in Table 4.2. It was found that the crystallinity degree of PCL in PCL/TCP(Si) with various GPTMS content is much higher than that in PCL/TCP. This may be due to the enhanced interfacial bonding between TCP(Si) and PCL, leading to higher nucleating efficiency of TCP(Si) for the crystallization of PCL. This increase in the crystallinity degree is correlated with the increase in compressive modulus and strength [134]. However, the melting temperature seemed to be independent of the introduction of GPTMS.

Table 4.2 Thermal property of PCL/TCP(Si) composites determined by DSC

<table>
<thead>
<tr>
<th>wt% of GPTMS to TCP</th>
<th>Melting temperature (°C)</th>
<th>Crystallinity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>55.3</td>
<td>39.9</td>
</tr>
<tr>
<td>5</td>
<td>55.0</td>
<td>47.2</td>
</tr>
<tr>
<td>10</td>
<td>55.1</td>
<td>47.7</td>
</tr>
<tr>
<td>15</td>
<td>54.8</td>
<td>46.4</td>
</tr>
</tbody>
</table>

Figure 4.6 SEM images of (a) PCL/TCP and (b) PCL/TCP(Si) scaffolds.

The surface morphology of PCL/TCP(Si), PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin scaffolds was characterized by using SEM. As shown in Figure 4.6 both PCL/TCP and PCL/TCP(Si) scaffolds exhibit smooth surface morphology.

4.2.2.2 In vitro cells response

The cell-scaffold interaction is crucial for tissue engineering, and an ideal scaffold provides a suitable environment for cells to proliferate and differentiate. BMSCs have
self-renewal capability, multilineage differentiation potential and similar prospect as embryonic stem cells, but they are less related with ethical issues and tumorigenesis risks [135]. Therefore, BMSCs have been widely employed for *in vitro* study of bone formation and evaluating the biocompatibility of a newly developed material for bone tissue engineering [135-137]. In this study, the biocompatibility of the developed PCL/TCP(Si) scaffolds are evaluated by observing BMSCs morphology, proliferation, and gene and protein expression.

Figure 4.7 SEM images of cell-scaffold construct of (a) PCL/TCP and (b) PCL/TCP(Si) scaffolds.

![Figure 4.7 SEM images of cell-scaffold construct of (a) PCL/TCP and (b) PCL/TCP(Si) scaffolds.](image)

Figure 4.8 Confocal laser microscopy with depth projection images reconstructed from multiple horizontal images, showing 3D distribution of cells within the scaffolds of PCL/TCP and PCL/TCP(Si) scaffolds.

![Figure 4.8 Confocal laser microscopy with depth projection images reconstructed from multiple horizontal images, showing 3D distribution of cells within the scaffolds of PCL/TCP and PCL/TCP(Si) scaffolds.](image)
Figure 4.7 shows the SEM images of BMSCs cultured on PCL/TCP and PCL/TCP(Si) scaffolds at day 7. For both PCL/TCP and PCL/TCP(Si) scaffolds the cells appeared to spread almost in the same manner.

Figure 4.8 shows the confocal laser microscopy image of BMSCs cultured on PCL/TCP and PCL/TCP(Si) scaffolds at different culture time. With predominantly in round shape, at day 10, for both of the groups cells have grown almost in similar manner in all the time points.

Figure 4.9 PicoGreen® DNA quantification results of BMSCs cultured on PCL/TCP and PCL/TCP(Si) scaffolds.

Figure 4.9 shows the proliferation of BMSCs in the cell-scaffold constructs studied by PicoGreen® assay. At all the time points, PCL/TCP(Si) scaffolds exhibited almost similar amount of DNA content like PCL/TCP scaffolds. The differentiation of BMSCs in the cell-scaffold constructs was studied by RT-PCR analysis. The quantitative RT-PCR analysis (Figure 4.10) revealed that PCL/TCP(Si) exhibited higher amount of Cbfa1 in both of the days 17 and 24 compared to PCL/TCP scaffolds. Cbfa1 which is
considered as an important transcription factor for the commitment of multipotent mesenchymal cells into the osteoblastic lineage by triggering the gene expression of bone matrix proteins [138, 139]. On the other hand, (OCN) which is one of the terminal differentiation markers and collagen I (Col1) which relates to further BMSCs differentiation to osteoblast, both showed slightly basal expression at day 17 and 24.

To analyze the extracts of protein formed on cell-scaffold constructs western blotting was carried out after BMSCs were cultured for 31 days. As can be seen in Figure 4.11 osteonectin (ON) which is an important non-collagen calcium binding glycoprotein and osteopontin (OPN) which is another mineral-binding protein found in bone extracellular matrix are almost in the same level for PCL/TCP and PCL/TCP(Si). Whereas, bone-specific glycoprotein OCN is 1.2 times higher amount for PCL/TCP(Si).

All these *in vitro* studies demonstrated that PCL/TCP(Si) scaffolds possess the same level of biocompatibility like PCL/TCP scaffolds, indicating GPTMS modification on TCP does not lead to any detrimental effect on BMSCs activity as also reported previously on the biocompatibility of silane treated surface [138, 140, 141].
Figure 4.10 mRNA expression of Cbfa1, Collagen I (Col1) and Osteocalcin (OCN) of BMSCs cultured for 17 days and 24 days on PCL/TCP and PCL/TCP(Si) scaffolds.
Additive manufactured PCL/TCP scaffolds with enhanced mechanical properties have been successfully prepared. TCP was modified by using GPTMS and the modified TCP has been incorporated into PCL to form reinforced PCL/TCP(Si) composite thorough homogenization followed by melt compounding and annealing at 120 °C for 2 hours. The successful modification of TCP was verified by XPS and FTIR analysis. Additive manufactured PCL/TCP(Si) scaffolds with 100% interconnectivity and 65% porosity were fabricated using an in-house SES. The PCL/TCP(Si) scaffolds showed 6.0 times higher compressive modulus and 2.3 times higher compressive strength than those of PCL/TCP scaffolds and eventually falls within range of the mechanical properties (stiffness 50 to 500 MPa and strength 3 to 12 MPa) of cancellous bone. Moreover, no negative effect of GPTMS modification was noticed.
4.3 Part II – POSS modified PCL/TCP(Si): composite synthesis, scaffolds fabrication and characterization

4.3.1 Materials and methods

4.3.1.1 Materials

Tri-silanollsobutyl POSS was purchaged from Hybrid Plastics, USA. PCL and TCP were purchased from Sigma-Aldrich, Singapore and Progentix, The Netherlands, respectively as mentioned in part I.

4.3.1.2 Synthesis of PCL/TCP(POSS)

PCL, TCP and POSS were melt compounded at 120 ºC for 20 min with 2, 4, 6, 8 and 10% of POSS composition referred to PCL/TCP composite. To ensure successful bonding between PCL, TCP and POSS, the dried POSS modified PCL/TCP (PCL/TCP(POSS)) composite was further modified through annealing at 180 ºC for 2 hr. After synthesizing the composites, scaffolds were fabricated using an in-house SES.

4.3.1.3 Fabrication of the PCL/TCP(POSS) scaffolds by SES

The scaffolds were fabricated as described in the part I of this chapter.

4.3.1.4 Characterization

Compressive modulus and compressive strength of the scaffolds were measured using an Instron 4502 uniaxial testing system as described in the part I of this chapter. Here, compression testing was done in both dry and simulated physiological test. Scaffolds were immersed in phosphate buffer solution (PBS) for 24 hr at 37 ºC before conducting compression testing in physiological condition.
The surface morphology and chemical structure of the scaffolds were characterized by FESEM and XPS as described in the part I of this chapter.

The processibility of PCL/TCP(POSS) composite was evaluated by a bench top capillary rheometer, Rosand 2000 (Malvern, USA) using a 1 mm diameter die. The composite was processed at 85 ºC and processibility was evaluated in terms of shear viscosity.

4.3.1.5 Cell seeding on scaffolds

Rat BMSCs (1.8×10^5 cells) were added onto each of the PCL/TCP and PCL/TCP(POSS) scaffold according to the protocol described in the in vitro cell culture study section of chapter 3. The growth media of the cell-scaffold constructs was supplemented with an osteogenic cocktail of dexamethasone (10 nM), ascorbic acid (50 μM) and β-glycerolphosphate (10 mM) (Sigma) throughout the experiment.

4.3.1.6 Morphology of the cell-scaffold constructs

SEM and confocal laser microscopy were used to assess cell morphology, viability and attachment in vitro according to the protocol described in the in vitro cell culture study section of chapter 3.

4.3.1.7 PicoGreen® Assay

Proliferation of BMSCs in cultured cell-scaffold constructs were studied using PicoGreen® assay as described in part I of this chapter.
Chapter 4.

4.3.1.8 Alkaline phosphate activity (ALP)

Osteogenic differentiation of the cells was measured using ALP biomarker. At predetermined time-points, cell-scaffold constructs were cut into smaller pieces and incubated in radio-immuno precipitation assay (RIPA) buffer for 10 min at 4 °C to allow lysis of cells. The constructs were then briefly vortexed and the lysate re-located into 1.5 mL Eppendorf tubes. The lysates were centrifuged at 13.2 K rpm for 10 min at 4 °C to separate the supernatant from cellular and tissue debris. Amount of total protein in each lysate was quantified using BCA Protein Assay kit (Thermo Scientific, #23225) according to manufacturer's protocol. Equal amount of total protein was loaded for every sample into a 96 multi-well plate. The protein solution was then incubated with a solution of pNPP Substrate for Alkaline Phosphatase (Invitrogen, #00-2212) for 60 min at 37 °C. Absorbance of the resulting solution was read at 405 nm using a Victor3 1420 Multi label Counter (Perkin Elmer).

4.3.1.9 Statistical Analysis

All the data presented are expressed as mean ± standard deviation. An unpaired student’s t-test was used to test the significance level of the data. Differences were considered statistically significant at p<0.05.

4.3.2 Results and Discussions

4.3.2.1 PCL/TCP(POSS) composite preparation and scaffolds fabrication

As a continuation effort to prepare additive manufactured PCL/TCP scaffolds with adequate mechanical property for bone tissue engineering, POSS has been used as a coupling agent to improve the interfacial bonding between PCL and TCP. POSS is a
hybrid (organic–inorganic) nanostructured macromere and has been used extensively in
the field of polymeric nanocomposites [142-144]. The incorporation of POSS derivatives
into polymeric materials can lead to different improvements in polymer properties
including mechanical properties and processibility [145, 146]. In recent years it has been
shown that POSS are biocompatible and have a great potential in the field of tissue
engineering [147, 148]. In this context, POSS has been used to modify PCL/TCP
composite. The hydroxyl groups of POSS are capable of reacting with the carboxyl end
groups of PCL, thus covalently binding the nanostructured moiety to the polymer [149].
To ensure this covalent bonding PCL/TCP(POSS) composite was annealed at 180 °C.
Similar reaction has been mentioned elsewhere [145].

Additive manufactured PCL/TCP(POSS) scaffolds were fabricated from the
synthesized PCL/TCP(POSS) composite using an in-house SES with pore size of 750-
950 μm and overall porosity of 65%. PCL/TCP scaffolds with the same dimension were
also fabricated as control. The compressive modulus and strength of PCL/TCP(POSS)
scaffolds with varying content of POSS were measured by compression testing, and
compared with those of PCL/TCP scaffold both in dry and simulated physiological state.
As shown in Figure 4.12, both compressive modulus and strength of PCL/TCP(POSS)
scaffolds are significantly higher than those of PCL/TCP scaffold, and PCL/TCP(POSS)
scaffold with 4 wt% of POSS referred to PCL/TCP composite exhibits the maxima
compressive modulus of 93.8 MPa and compressive strength of 6.0 MPa, which are 6.9
and 3.5 times of those of PCL/TCP scaffolds, respectively. The compressive modulus of
PCL/TCP decreased by 15% in simulated physiological state compared to dry state,
whereas except 10 wt% POSS modified scaffolds all other POSS modified scaffolds
reduced by less than 10%. This result indicates the capability of POSS modified
scaffolds to retain mechanical properties in simulated physiological condition. Among all
the POSS modified groups 4 wt% POSS modified PCL/TCP scaffolds showed best mechanical properties, hence the rest of the characterizations and \textit{in vitro} evaluation was carried out on this group of scaffolds and notified by PCL/TCP(POSS) scaffolds.

Figure 4.12 Compressive modulus (a) and (b) compressive strength of PCL/TCP and PCL/TCP(POSS) scaffolds with various content of POSS referred to PCL/TCP composite. All POSS modified groups are statistically significant (*p<0.05) compared to PCL/TCP alone.
The surface morphology of PCL/TCP and PCL/TCP(POSS) scaffolds was characterized by using SEM. As shown in Figure 4.13 PCL/TCP scaffolds exhibit smooth surface morphology, whereas PCL/TCP(POSS) showed exposed TCP. During polymer ceramic blending, generally ceramic particles are covered by polymer during mixing, and hence the bioactivity of the ceramic particles reduced [150]. In this respect the findings of exposed TCP on the scaffolds surface is quite interesting. To confirm the presence of exposed TCP, XPS was carried out. As shown in Figure 4.14, while only carbon and oxygen are detected on the surface of PCL/TCP scaffolds, additional calcium and phosphorous peaks are observed in the XPS spectrum of PCL/TCP(POSS) scaffolds, confirming the presence of exposed TCP on the surface of the scaffolds.

As can be seen from Figure 4.15 shear viscosity reduces with the increase of POSS addition. This signifies that POSS can also be used to improve the processibility of PCL/TCP composite. Similar phenomenon has been noticed while POSS were blended with poly (lactic acid) (PLA) [151].

Figure 4.13 SEM images of (a) PCL/TCP and (b) PCL/TCP(POSS) scaffolds.
Figure 4.14 XPS spectra of (a) PCL/TCP and (b) PCL/TCP(POSS) scaffolds.

Figure 4.15 Shear viscosity vs. shear rate of PCL, PCL/TCP and PCL/TCP(POSS) with various content of POSS referred to PCL/TCP.
4.3.2.2 In vitro cells response

In this study, the biocompatibility of the developed PCL/TCP(POSS) scaffolds are evaluated by observing rat BMSCs morphology, proliferation, and ALP expression.

Figure 4.16 shows the SEM images of BMSCs cultured on PCL/TCP and PCL/TCP(POSS) scaffolds at day 14 and 21. At day 21, cells on PCL/TCP appeared to spread in a limited manner compared to PCL/TCP(POSS) scaffolds. Moreover, cells on PCL/TCP(POSS) scaffolds showed apparently better contact with surface compared to PCL/TCP scaffolds.

Figure 4.17 shows the confocal laser microscopy image of BMSCs cultured on PCL/TCP and PCL/TCP(POSS) scaffolds at day 14 and 21. At day 21, PCL/TCP(POSS) scaffolds showed uniform distribution of cells compared to PCL/TCP scaffolds, and thus the confocal imaging result is in line with the SEM result.

Figure 4.18 shows the proliferation of BMSCs in the cell-scaffold constructs studied by PicoGreen® assay. At day 14, PCL/TCP(POSS) scaffolds exhibited around 1.3 times higher amount of DNA content compared to PCL/TCP scaffolds. However, no statistically significant result has been noticed.

To measure the osteogenic differentiation of BMSCs ALP expression has been measured. It has been used as measuring ALP expression is straightforward, reliable and less expensive. As can be seen from Figure 4.19 PCL/TCP(POSS) scaffolds exhibited greater amount of ALP expression compared to PCL/TCP scaffolds. At day 21 and day 28, PCL/TCP(POSS) scaffolds exhibited almost 4.4 and 2.1 times higher amount of ALP expression compared to PCL/TCP scaffolds. The cells on PCL/TCP(POSS) scaffolds get contact with the exposed TCP, and therefore, they are more line to the osteogenic lineage
compared to the cells on PCL/TCP scaffolds. Previous study also showed that calcium phosphate surface promotes osteogenic differentiation to BMSCs [152].

Figure 4.16 SEM images of cell-scaffold construct of PCL/TCP and PCL/TCP(POSS) scaffolds at day 14 and day 21.

Figure 4.17 Confocal laser microscopy with depth projection images reconstructed from multiple horizontal images shows 3D distribution of cells within the scaffolds of PCL/TCP and PCL/TCP(POSS).
Figure 4.18 PicoGreen® DNA quantification results of rat BMSCs cultured on PCL/TCP and PCL/TCP(POSS) scaffolds (*p<0.05).

Figure 4.19 Alkaline phosphate expression normalized to protein content of rat BMSC seeded PCL/TCP and PCL/TCP(POSS) scaffolds (*p<0.05).
All these *in vitro* studies demonstrated that PCL/TCP(POSS) scaffolds are suitable for bone tissue engineering, as we can also found from previous studies [147, 148]. Moreover, our study suggests that PCL/TCP(POSS) scaffolds may be more beneficial due to the exposed TCP particles on the surface of the scaffolds.

### 4.3.3 Conclusions

In this study POSS modified PCL/TCP scaffolds with enhanced mechanical properties have been successfully prepared. PCL/TCP(POSS) scaffolds showed 6.9 times higher compressive modulus and 3.5 times higher compressive strength than those of PCL/TCP scaffolds and eventually falls within range of the mechanical properties (stiffness 50 to 500 MPa and strength 3 to 12 MPa) of cancellous bone. This study also suggests that POSS can be used not only to improve the mechanical properties but also the processibility of PCL/TCP scaffolds. Due to the change of viscoelastic properties of the POSS modified scaffolds TCP has been exposed to the surface. This exposed TCP may improve the biocompatibility of the POSS modified PCL/TCP scaffolds. This claim can be supported from the findings of SEM, confocal images and ALP activity of *in vitro* study.

In this chapter two different types of coupling agent namely, silane and POSS have been used successfully to improve the mechanical properties of the PCL/TCP scaffolds. Both of the silane and POSS modified PCL/TCP scaffolds have significantly improved mechanical properties compared to control PCL/TCP scaffolds, and are suitable to be used for cancellous bone TE applications. However, in terms of biocompatibility and mechanical properties POSS modified scaffolds showed better performance compared to
silane modified scaffolds. Eventually this study extends the potential of melt extrusion based additive manufactured composite scaffolds for bone tissue engineering.

However, a scaffold should not only have adequate mechanical properties but also have functional properties like improved biocompatibility and cells entrapment system. In the subsequent chapters, silane and POSS modified PCL/TCP scaffolds will be used for further modification to improve the biocompatibility and cells entrapment efficiency of the scaffolds, respectively. To improve the proliferative and osteoconductive properties of the scaffolds biomimetic composite coating has been developed on PCL/TCP(Si) scaffolds which will be discussed in the next chapter.
Chapter 5

Development of Biomimetic Composite Coating on PCL/TCP(Si) scaffolds

5.1 Introduction

Polymer/ceramic composite scaffolds are preferable candidates for bone tissue engineering because they integrate the favourable properties of both the polymer and ceramic, and hence enhance mechanical properties and biocompatibility [12, 14, 128]. However, blending ceramic with polymer directly may lead to masking of the ceramic particles with polymer [150]. This limits exposure of ceramics on the scaffold surface and hinders the direct contact of cells to the bioactive ceramics particles, and thus diminishes the osteoconductive property offered by the ceramic particles. Therefore, in order to improve the osteoconductive property of the polymer/ceramic composite scaffolds, a layer of ceramic coating on the scaffold’s surface is considered as an efficient approach. It should be noted that ideal surface coating can provide desirable biological properties to the bulk implant, whilst retains the structural properties of the scaffolds.

Among various coating approaches, the biomimetic approach acts in a more efficient and similar way to the natural system [153]. Kokubo et al. first reported the use of simulated body fluid (SBF) for biomimetic growth of apatite coatings on bioactive CaO-SiO₂ glass by mimicking the natural biomineralization process [154]. However, this conventional biomimetic process exhibits some limitations, notably the time consuming nature of the process which may take several weeks [155, 156]. Though the process time
could be shortened by using concentrated SBF, it still requires a constant pH and replenishing the concentrated SBF frequently to maintain supersaturation for apatite crystal growth [157]. Therefore, an alternative, simpler and more efficient approach for biomimetic coating is required.

Previous study on AM scaffolds coated with apatite did not find any promising result due to flaking of the thick apatite layer on the scaffolds [113]. To reduce the flaking tendency composite coating may be desirable, as a recent study proved that collagen can promote both the cohesive and adhesive properties of apatite coating on titanium with a significantly higher coating retention for the apatite-collagen composite coating compared to apatite coating [158]. However, relatively little work has been reported on apatite-collagen composite coating on polymer related scaffolds [159]. More specifically there is no study of such composite coating on AM scaffolds.

In comparison to collagen, collagen-based gelatin has a higher degree of biological functional groups and, as gelatin is denatured, it overcomes the possible concerns of immunogenicity associated with collagen. Moreover, its cost efficiency can facilitate its selectivity over collagen. Previous studies have reported the use of hydroxyapatite (HA)-gelatin composite as a scaffold for bone tissue engineering [160-162]. It was found that stimulation of osteoblast responses were significantly higher on co-precipitated HA-gelatin scaffolds than on pure gelatin or conventional HA-gelatin scaffolds prepared by directly mixing the gelatin with HA [161]. However, biological apatites are always carbonated from a compositional point of view. Carbonated hydroxyapatite (CHA) implants exhibit higher osteoconductive properties and earlier bioresorption compared to HA samples [163]. Therefore, we hypothesize that the combination of CHA and gelatin
as a composite coating through biomimetic co-precipitation has the potential to be an effective biomimetic coating for bone tissue engineering.

The objective of the present study is to develop a facile but efficient approach to provide a biomimetic composite coating on AM scaffolds for bone TE. CHA-gelatin composite coating was formed on previously developed PCL/TCP(Si) scaffolds through biomimetic co-precipitation. CHA coated scaffolds were also prepared to understand the influence of gelatin incorporation on cell proliferation and differentiation activity. The scaffolds were evaluated for surface morphology, composition and compressive modulus. The biological capabilities of these scaffolds were also evaluated by culturing porcine bone marrow stromal cells (BMSCs) on the scaffolds.

5.2 Materials and methods

5.2.1 Materials

Poly(ε-caprolactone) (PCL) ($M_n$: 80,000), gelatin (type A, from porcine skin), calcium chloride (CaCl$_2$), phosphoric acid (H$_3$PO$_4$, 85% solution in water), sodium carbonate (Na$_2$CO$_3$), acetic acid (CH$_3$COOH), sodium hydroxide (NaOH), and potassium hydrophosphate (K$_2$HPO$_4$) were purchased from Sigma-Aldrich (Singapore). Tri-calcium phosphate (TCP) was obtained from Progentix (The Netherlands).

5.2.2 Fabrication of PCL/TCP(Si) scaffolds by SES

PCL/TCP(Si) scaffolds were developed and fabricated according to the process described in the materials and methods of chapter 4.
5.2.3 Surface coating on PCL/TCP scaffolds

PCL/TCP(Si) scaffolds were first treated in 10 ml 5 M NaOH at room temperature for 12 hours followed by thorough washing with de-ionized water to remove the free NaOH. The NaOH treated scaffolds were then dipped alternately into calcium chloride solution and potassium hydrophosphate solution to obtain CaHPO$_4$ coating as a nucleation site for the next CHA coating or CHA-gelatin composite coating [111]. In brief, the NaOH treated scaffolds were dipped in 20 mL 0.2 M CaCl$_2$ aqueous solutions for 10 minutes and then dipped in de-ionized water for 5 seconds followed by air drying for 3 minutes. The sample was subsequently dipped in 20 mL 0.2 M K$_2$HPO$_4$ aqueous solutions for 10 minutes and then dipped in de-ionized water for 5 seconds followed by air drying for 3 minutes. The whole process was repeated three times.

The CaHPO$_4$ coated scaffolds were immersed into 20 mL 0.1 M CH$_3$COOH, and then 10 ml 0.1 M CaCl$_2$ and 6 ml 0.1 M H$_3$PO$_4$ (Ca/P = 1.66) were dropped slowly through separate syringe pumps under stirring. The pumps were adjusted to keep the ratio of Ca/P at 1.66. After further stirring for 30 min, 18 ml 0.1 M Na$_2$CO$_3$ with the molar ratio of CO$_3^{2-}$ / PO$_4^{3-}$ = 3 was dropped gradually. The mixture was stirred for another 30 min and then, the pH of the mixture was adjusted to 9 using 0.1 M NaOH. The CHA coated PCL/TCP(Si) scaffolds (PCL/TCP(Si)-CHA) were collected after aging the solution for 3 hr. Finally, the scaffolds were washed with de-ionized water thoroughly and freeze dried.

For preparing CHA-gelatin composite coated PCL/TCP(Si) scaffolds (PCL/TCP(Si)-CHA-gelatin), first 40 mg gelatin was dissolved into 20 ml de-ionized water at 37 °C to get gelatin solution (2 mg/mL). Then CaHPO$_4$ coated scaffolds were dipped into the above gelatin solution for 0.5 hr followed by washing with de-ionized
water to remove free gelatin. The gelatin coated scaffolds were then immersed into 20 ml 0.1 M CH₃COOH solution dissolved with 40 mg gelatin. Finally CHA-gelatin composite was coated onto the gelatin treated PCL/TCP(Si) scaffolds by adding CaCl₂ and H₃PO₄ following the same process as that of CHA coating.

5.2.4 Scaffolds characterizations

The surface morphology of scaffolds was observed with a field emission scanning electron microscope (SEM) (Philips XL30 FEG) according to the methods described in the materials and methods of chapter 4. The thickness of CHA coating and CHA-gelatin composite coating were measured from the cross-section SEM image of filament in coated scaffolds. Attenuated total refraction Fourier transform infrared spectroscopy (ATR-FTIR) analysis of the coating was performed on an Avatar 380 (Thermo Nicolet) over a range of 800-1800 cm⁻¹ at resolution of 2 cm⁻¹ to study the chemical structure of the coating. ATR-FTIR testing was carried out with film samples assuming that coatings on both film and scaffolds were similar. The tested films were prepared by hot pressing PCL/TCP(Si) composite and followed by the same coating process as that with scaffolds. XPS (Thetaprobe from Thermo Scientific) was used to study the scaffold surface according to the method described in the materials and methods of chapter 4.

5.2.5 Compression testing

Compression testing of the scaffolds was conducted by using an Instron 4502 uniaxial testing system according to the protocol described in the materials and methods of chapter 4. Compression testing was also carried out under simulated physiological condition by keeping the scaffolds at 37 °C in phosphate buffer saline (PBS) solution for 24 hours before testing.
5.2.6 Cell seeding on scaffolds

Three groups of scaffolds were prepared namely, PCL/TCP(Si), PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin and each scaffold was seeded with approximately $1.8 \times 10^5$ BMSCs according to the protocol described in the materials and methods of part I chapter 4.

5.2.7 Morphology of the cell-scaffolds constructs

Confocal laser microscopy and SEM were used to assess cell viability, morphology and attachment in vitro as described in the materials and methods of part I chapter 4.

5.2.8 PicoGreen® assay

Proliferation of BMSCs in cell-scaffold constructs were studied using PicoGreen® assay as per manufacturer’s protocol (Molecular Probes, PicoGreen dsDNA Quantitation kit) was described in the materials and methods of part I chapter 4.

5.2.9 Reverse transcription polymerase chain reaction (RT-PCR)

Osteogenic gene expression of BMSCs at in vitro culture were analysed at day 17 and 24. The protocol was described in the materials and methods of part I chapter 4.

5.2.10 Western Blotting (WB)

Osteogenic proteins of BMSCs at in vitro culture were analysed at day 17 and 24. The protocol was described in the materials and methods of part I chapter 4.
5.2.11 Statistical analysis

An unpaired student’s t-test was performed to evaluate the significance of observed differences between the study groups. A value of $p<0.05$ was considered to be statistically significant. Data were tested for normality prior to performing the student’s t-test.

5.3. Results and discussion

5.3.1 Scaffolds fabrication

In the present study bioresorbable PCL/TCP(Si) scaffolds were fabricated using SES with the following structural properties: i) 100% pore interconnectivity with pore size of $\sim 500 \mu m$; ii) scaffold porosity of 65%; and iii) $0^\circ/60^\circ/120^\circ$ lay down pattern. The architectural layout of the developed scaffolds resembles the honeycomb which is advantageous over foam type structure. It has been reported that honeycomb structure allows bone ingrowths at later stages [164, 165], whereas foam structure limits tissue growth confined to the surface of the foam [166, 167].

5.3.2 Biomimetic CHA-gelatin composite coating

In order to improve the osteoconductive property of additive manufactured PCL/TCP(Si) scaffolds, CHA-gelatin composite was coated onto additive manufactured PCL/TCP(Si) scaffolds through a facile biomimetic process. Scaffolds coated with CHA alone were also prepared to verify the improvement on the performance of scaffolds due to the incorporated gelatin. To promote biomimetic mineralization on the surface of PCL/TCP(Si) scaffolds in CHA coating or CHA-gelatin composite coating, CaHPO$_4$ was
coated onto the scaffold first for inducing surface nucleation as reported previously [111]. In the preparation of CHA coated PCL/TCP(Si) scaffolds, the CaHPO$_4$ coated PCL/TCP(Si) scaffolds were immersed into CH$_3$COOH followed by simultaneously dropping CaCl$_2$ and H$_3$PO$_4$, and further dropping Na$_2$CO$_3$ according to the approach for CHA preparation as reported previously [168]. Due to the nucleation of CaHPO$_4$, CHA was coated onto the PCL/TCP(Si) scaffolds. As to CHA-gelatin composite coated PCL/TCP(Si) scaffolds (PCL/TCP(Si)-CHA-gelatin), the CaHPO$_4$ coated PCL/TCP(Si) scaffolds were further coated with gelatin via electrostatic attraction between CaHPO$_4$ and gelatin by dipping into gelatin solution [169]. Finally, gelatin was co-precipitated with CHA on scaffold surface to produce CHA-gelatin composite coating via the biomimetic process similar as that for CHA coating. The carboxyl and amine groups present in the gelatin may become charged groups under the coating condition, such as –COO$^-$ and –NH$_3^+$, which can promote CHA-gelatin composite nucleation. It should be noted that the whole approach is free from gelatin cross-linking. Thus, the potential toxicity caused by chemicals employed for cross-linking gelatin in other systems can be avoided [170, 171]. More interestingly, with the biomimetic approach described here, biomineralized coating can be achieved in a few hours. Figure 5.1 shows the flow diagram of the composite coating process.
5.3.3 Scaffold characterization

PCL/TCP(Si), PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin scaffolds were characterized using SEM, ATR-FTIR and XPS. Compression testing was also conducted to determine whether there is any detrimental effect of the coating process on compressive modulus of the scaffolds.

Figure 5.2 SEM images of (a) PCL/TCP(Si), (b) PCL/TCP(Si)-CHA and (c) PCL/TCP(Si)-CHA-gelatin scaffolds.
The surface morphology of PCL/TCP(Si), PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin scaffolds was characterized by using SEM. As shown in Figure 5.2, while the PCL/TCP(Si) scaffolds exhibit smooth surfaces, both the PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin scaffolds exhibit rough surfaces with uniform coating throughout. The CHA coating looks like globular apatite (Figure 5.2b) and CHA-gelatin composite coating looks like plate-like apatite (Figure 5.2c). The morphology difference between CHA coating and CHA-gelatin composite coating could be due to the presence of gelatin in the biomimetic co-precipitation for CHA-gelatin composite coating, as it is known that adsorption of protein on the mineral surface can alter the nucleation and growth of crystal in biomineralization [172]. The thickness of CHA coating and CHA-gelatin composite coating are measured as 702 ± 56 nm and 635 ± 119 nm from their cross-section SEM images, respectively (Figure 5.3).

![Figure 5.3 SEM image of the cross-section of filament in (a) PCL/TCP(Si)-CHA and (b) PCL/TCP(Si)-CHA-gelatin scaffolds.](image)
The chemical structure of CHA-gelatin composite coating was studied using ATR-FTIR. Figure 5.4 shows the FTIR spectrum of pure gelatin with the characteristic amide I and II bands at 1650 and 1550 cm\(^{-1}\), respectively. The observation of these characteristic amide bands of gelatin in the ATR-FTIR spectrum of CHA-gelatin composite coated PCL/TCP films reveals the presence of gelatin in the composite coating (Figure 5.4b). In the ATR-FTIR spectra of both CHA coated and CHA-gelatin composite coated PCL/TCP films, the characteristic bands for phosphate and carbonate groups were observed at 1039 cm\(^{-1}\) and 1400 cm\(^{-1}\), respectively, which are similar to those found in natural bone [150, 173]. This demonstrates the formation of CHA with bone like chemical composition in the coating.
The chemical structure of the coating was also studied by using XPS. No characteristic peak of calcium and phosphorous is detected in the XPS spectrum of PCL/TCP(Si) scaffolds as shown in Figure 5.5. This implies that ceramic particles of TCP(Si) are covered by PCL during composite preparation and/or scaffolds fabrication as reported previously [150]. In contrast, characteristic peaks of calcium and phosphate are observed in the XPS spectra of both PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin scaffolds. Moreover, a nitrogen peak is detected in the XPS spectrum of PCL/TCP(Si)-CHA-gelatin scaffolds (Figure 5.5c). All these confirm the successful formation of CHA and CHA-gelatin coating on the strut surface of PCL/TCP(Si) scaffolds.
Figure 5.6 Compression modulus (a) and compressive strength (b) of PCL/TCP(Si), PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin scaffolds in dry and simulated physiological state.

One of the basic functions of scaffolds for bone tissue engineering is to act as a supporting structure and provide adequate mechanical strength to maintain the spaces required for cell growth in physiological condition. The developed PCL/TCP(Si) scaffolds have adequate mechanical properties to be used in cancellous bone tissue
engineering application. However, compression testing on coated PCL/TCP(Si) scaffolds was carried out to evaluate whether there is any detrimental effect of the biomimetic coating process on mechanical properties. Compression testing in dry state and simulated physiological state revealed that PCL/TCP(Si)-CHA scaffolds and PCL/TCP(Si)-CHA-gelatin scaffolds have almost the same mechanical properties like PCL/TCP(Si) scaffolds [Figure 5.6], which signifies no detrimental effect of coating process on mechanical properties of the PCL/TCP(Si) scaffolds both in dry state and simulated physiological state.

5.3.4 In vitro cells response

In this study, the proliferative and osteoconductive properties of the developed CHA-gelatin composite coating on PCL/TCP(Si) is evaluated by observing BMSCs morphology, proliferation, and gene and protein expression. Figure 5.7 shows the SEM images of BMSCs cultured on PCL/TCP, PCL/TCP(Si), PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin scaffolds at day 7. Highest level of cells and cell-sheet accumulation were observed on PCL/TCP(Si)-CHA-gelatin scaffolds among all the scaffolds. Moreover, from the overview image of the PCL/TCP(Si)-CHA-gelatin scaffolds it can be seen that tissue bridges have started to form (indicated by arrow in Figure 5.8).

Figure 5.7 SEM images of cell-scaffold construct of (a) PCL/TCP(Si), (b) PCL/TCP(Si)-CHA and (c) PCL/TCP(Si)-CHA-gelatin scaffolds at day 7. The arrow shows the cells and/or cell-sheet accumulation that have spread on surface of the scaffolds.
Figure 5.8 SEM image of cell-scaffold construct of PCL/TCP(Si)-CHA-gelatin scaffolds at day 7. The arrow shows starting of tissue bridge formation.

Figure 5.9 shows the confocal laser microscopy image of BMSCs cultured on PCL/TCP(Si), PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin scaffolds at different culture time. At day 7, BMSCs stretched well over the struts and distributed uniformly in PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin scaffolds, while qualitatively fewer BMSCs were observed in non-coated PCL/TCP(Si) scaffolds. At day 10, PCL/TCP(Si)-CHA-gelatin scaffolds exhibited tissue bridges between the struts (Figure 5.10) as well as the most uniform distribution of viable cells among all the scaffold groups (Figure 5.9). In contrast, at day 10, the BMSCs seeded in PCL/TCP(Si) scaffolds were predominantly in round shape. It should be noted that our developed thin CHA-gelatin composite coating did not show any flaking tendency during cell culture as opposed to previous study [113], demonstrating excellent adhesion of the biomimetic composite coating to the scaffolds.
Figure 5.9 Confocal laser microscopy with depth projection images reconstructed from multiple horizontal images, showing 3D distribution of cells within the scaffolds of PCL/TCP(Si), PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin scaffolds.

Figure 5.10 Confocal laser microscopy image of PCL/TCP(Si)-CHA-gelatin scaffolds at day 10, showing tissue bridge as indicated by the arrow.
Figure 5.11 shows the proliferation of BMSCs in the cell-scaffold constructs studied by PicoGreen® assay. At all the time points, coated PCL/TCP(Si) scaffolds exhibit higher amount of DNA in comparison to non-coated PCL/TCP and PCL/TCP(Si) scaffolds. At day 7, PCL/TCP(Si)-CHA-gelatin scaffolds exhibit significantly higher amount of DNA in comparison to non-coated PCL/TCP(Si) scaffolds. At day 10, both PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin scaffolds exhibit significantly higher amount of DNA in comparison to non-coated PCL/TCP(Si) scaffolds, and the significantly highest amount of DNA content is exhibited by PCL/TCP(Si)-CHA-gelatin scaffolds among all the scaffolds, with 1.4 and 2.0 times higher value than that of PCL/TCP(Si)-CHA and PCL/TCP(Si) scaffolds, respectively. These findings signify the highest level of BMSCs proliferation on PCL/TCP(Si)-CHA-gelatin scaffolds among all the scaffold groups, which could be due to the presence of gelatin in CHA-gelatin composite coating. Gelatin is recognized to promote cell proliferation [161].
Figure 5.12 mRNA expression of Cbfa1, Collagen I (Col1) and Osteocalcin (OCN) of BMSCs cultured for 17 days and 24 days on PCL/TCP(Si), PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin scaffolds.

The differentiation of BMSCs in the cell-scaffold constructs was studied by RT-PCR analysis. The quantitative RT-PCR analysis (Figure 5.12) revealed that at day 17, PCL/TCP(Si)-CHA-gelatin and PCL/TCP(Si)-CHA scaffolds have 6.2 and 3.8 times
higher expression levels of Core binding factor α1 (Cbfa1) than that of PCL/TCP(Si) scaffolds, respectively. At day 24, which is a later time point, the Cbfa1 expression levels of PCL/TCP(Si)-CHA-gelatin and PCL/TCP(Si)-CHA scaffolds were 1.4 and 4.3 times higher than that of PCL/TCP(Si) scaffolds, respectively. Cbfa1 is considered as an important transcription factor for the commitment of multipotent mesenchymal cells into the osteoblastic lineage by triggering the gene expression of bone matrix proteins [138, 139]. Cbfa1 retains the osteoblastic cells at an immature stage by hampering the transition of osteoblasts to osteocytes. Generally, the expression of Cbfa1 is first noticed in preosteoblasts, and up regulated in immature osteoblasts, but down regulated in mature osteoblasts [139, 174]. Cbfa1 is also involved in regulating bone phenotypic genes such as osteocalcin (OCN), one of the terminal differentiation markers [139, 175, 176]. It can be seen from Figure 5.12, OCN is 25.2 and 9.8 times higher for PCL/TCP(Si)-CHA-gelatin and PCL/TCP(Si)-CHA scaffolds than that of the PCL/TCP(Si) scaffolds at day 24, respectively, when slightly lower expression level of Cbfa1 was observed. Expression of collagen I (Col1), which relates to further BMSCs differentiation to osteoblast, showed slightly basal expression levels for PCL/TCP(Si)-CHA-gelatin and PCL/TCP(Si)-CHA scaffolds compared to PCL/TCP(Si). Down regulation of Col1 expression of marrow stromal cells (MSC) on hydroxyapatite after day 10 has also been reported previously [152, 177].
To analyze the extracts of protein formed on cell-scaffold constructs western blotting was carried out after BMSCs were cultured for 31 days. Osteonectin (ON) is an important non-collagen calcium binding glycoprotein related to mineralisation at the early stage of bone formation secreted by osteoblasts. As can be seen from Figure 5.13, the expression of ON is 2.5 and 2.2 times higher on PCL/TCP(Si)-CHA-gelatin and PCL/TCP(Si)-CHA scaffolds compared to that on PCL/TCP(Si) scaffolds, respectively. OCN is a bone-specific glycoprotein found abundantly in bone and dentin. This non-collagenous protein is secreted by osteoblasts. It is known to promote calcification of the bone matrix, and has been studied as a late marker for osteogenic differentiation and osteoblast maturation [135, 139, 178]. The expression of OCN is 1.2 and 1.3 times higher on PCL/TCP(Si)-CHA-gelatin and PCL/TCP(Si)-CHA scaffolds than that on PCL/TCP(Si) scaffolds, respectively. Osteopontin (OPN) is another mineral-binding protein found in bone extracellular matrix and is associated with cell attachment,
proliferation, and biomineralization of extracellular matrix into bone. Its expression exhibits the commitment of BMSCs to osteogenic differentiation [178]. A significantly higher level (5.7 times) of OPN expression was observed on PCL/TCP(Si)-CHA-gelatin scaffolds compared to that on PCL/TCP(Si) scaffolds. At the same time, PCL/TCP(Si)-CHA scaffolds showed 4.7 times of OPN expression compared to PCL/TCP(Si) scaffolds. Figure 12 also showed a higher amount of ON and OPN expression on PCL/TCP(Si)-CHA-gelatin scaffolds than that on PCL/TCP(Si)-CHA scaffolds. Stimulation of osteogenic differentiation of BMSCs on PCL/TCP(Si)-CHA-gelatin scaffolds may be due to the presence of gelatin in CHA-gelatin composite coating [179]. Previous study on collagen and HA showed that the addition of collagen to hydroxyapatite implants can enhance both phagocytotic and osteogenic processes [180]. These results suggest that CHA-gelatin composite coating on PCL/TCP(Si) scaffolds improved the proliferation and osteoconductive capability of the scaffolds significantly.

5.4. Conclusions

Additive manufactured PCL/TCP(Si) scaffolds with improved osteoconductive properties have been successfully prepared by coating with CHA-gelatin composite via a biomimetic co-precipitation process and was verified by XPS analysis. SEM and confocal images demonstrated the highest level of cells and cell-sheet accumulation with most uniform distribution on PCL/TCP(Si)-CHA-gelatin scaffolds compared to all other scaffolds. The proliferation rate of BMSCs on CHA-gelatin composite coated PCL/TCP(Si) scaffolds were 2.0 and 1.4 times higher that of PCL/TCP(Si) and CHA coated PCL/TCP(Si) scaffolds, respectively, by day 10. In vitro RT-PCR and western blotting testing showed highest level of osteoconductive property on PCL/TCP(Si)-CHA-gelatin compared to all other scaffolds. These findings suggest that CHA-gelatin
composite coating is more effective than CHA coating on improving the proliferation and osteogenic differentiation of porcine BMSCs on additive manufactured scaffolds, which could be attributed to gelatin in the composite coating. This study provides a potential application of biomimetic composite coating on AM scaffolds. Consequently, the CHA-gelatin composite coated PCL/TCP(Si) scaffold could be regarded as a promising additive manufactured scaffold for bone tissue engineering.
Chapter 6

Development of Additive Manufacturing-Freeze Drying Integrated Scaffolds with POSS modified PCL/TCP Scaffolds

6.1 Introduction

Though extrusion based AM technology is a promising, straightforward and low cost process which has been used to fabricate complex scaffold architecture designs with controlled pore size which eventually results in efficient nutrient perfusion [11, 12, 181], the scaffolds fabricated with this technology still pose a limit in cell seeding efficiency and tissue formation due to pore resolution. The pore size between the two consecutive strands is too large as compared to the dimension of the cells which eventually results in poor cell seeding efficiency. To solve this problem different research groups have incorporated electrospun fibre network within the pore of the additive manufactured scaffolds [16, 107, 108, 182]. In this approach additive manufactured scaffolds offers structural integrity and electrospun network within the pores works as a cell entrapment system [16]. However, combining electrospinning process with extrusion based additive manufacturing process to fabricate integrated macro and micro fiber scaffolds is not very simple. In this hybrid process AM and electrospinning are done in a repetitive manner which requires a special experimental setup. Moreover, effort is needed to insulate the additive manufacturing robot from the electrospinning high voltage collector. Therefore, a simpler process to fabricate this type of hierarchical scaffolds needs to be developed.
Freeze drying technology in conjunction with porogen method can be considered as an easy and diverse porous hydrogel foam preparation process and has been used in pharmaceutical [183] and tissue engineering fields [184-186]. In this approach water is used as a solvent and ice is used as a porogenic template. The inner porous structure of the freeze dried hydrogel can be controlled by varying the concentration of the solution and also by varying the freeze drying conditions e.g freezing temperature [184] and freezing rate [187]. Aligned porous structure can also be created by unidirectional freeze drying [186].

Gelatin, which is a denatured derivative of collagen, has a high number of biological functional groups and has been used to produce drug carriers, wound dressing materials and scaffolds for tissue engineering [184, 188-190]. Porous gelatin scaffolds have been successfully prepared by dissolving in water followed by freeze drying [184]. Therefore, to use the freeze drying process to create porous gelatin structure within the large pores of the additive manufactured scaffolds will make the process of fabricating hierarchical scaffolds simpler.

Here, a novel route in fabricating hierarchical bone tissue engineering scaffolds has been designed with the combination and integration of AM and freeze drying technology. Firstly, POSS modified PCL/TCP composite was prepared, and then used to fabricate 3D porous extrusion based additive manufactured scaffolds. POSS modified PCL/TCP scaffolds have been shown earlier to provide significantly improved mechanical properties and are suitable to be used for cancellous bone tissue engineering application. Secondly, these 3D scaffolds were dipped into gelatin solution and porous gelatin structure was introduced within the pores of the 3D scaffolds through freeze drying. Porous gelatin structure has been cross-linked by using imide based zero-length
cross linker. Studies on cell-scaffold interaction were carried out by culturing rat bone marrow stromal cells (rBMSCs) on the scaffolds and assessing proliferation and differentiation. We hypothesize that the hierarchical scaffolds developed through this combinational approach will not only provide adequate mechanical properties but also improve the functional performance by improving cell seeding efficiency through the use of porous gelatin structure as a cell entrapment system.

6.2 Materials and methods

6.2.1 Materials

Poly(ε-caprolactone) (PCL) ($M_n$: 80,000), GPTMS, gelatin (type A, from porcine skin), sodium hydroxide (NaOH), acetic acid (CH$_3$COOH) and 1-ethyl-3-(3-dimehylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich, Singapore. Tri-calcium phosphate (TCP) was purchased from Progentix, The Netherlands. Tri silanollobutyl POSS was purchased from Hybrid Plastics, USA.

6.2.2 Fabrication of POSS modified PCL/TCP scaffolds by SES

POSS modified PCL/TCP composite was synthesized and PCL/TCP(POSS) scaffolds were fabricated according to the process described in chapter 4. Each layer of the scaffolds was fabricated with the designed pattern of 0º/60º/120º orientation. The size of the scaffolds used for the experiment was 5 mm × 5 mm × 3.5 mm.
6.2.3 Forming porous gelatin structure within the pores of the PCL/TCP(POSS) scaffolds and its characterization

PCL/TCP(POSS) scaffolds were immersed in aqueous NaOH solution (10 ml, 5 M) at room temperature for 1 hour followed by thorough washing with de-ionized water. The NaOH treated scaffolds were dipped in CH$_3$COOH (2 wt%) for 2 hours. The scaffolds were then dipped into 0.4 % (w/v) of gelatin solution for 2 hours at room temperature. Then the immersed scaffolds were freeze-dried at -80 °C for 24 hours, followed by freeze drying for 48 hours. The resulted porous gelatin structure within the 3D PCL/TCP(POSS)-foam scaffolds were cross-linked using an EDC/NHS agent based on the experimentally determined conditions: EDC (10 mL, 100 mM) and NHS (10 mL, 100 mM) in 95% ethanol for 24 hours at 4 °C.

PCL/TCP(POSS) scaffolds with gelatin porous structure within the pores of it will be named as PCL/TCP(POSS)-foam scaffolds.

6.2.4 Cell seeding on scaffolds

Two groups of scaffolds were prepared namely, PCL/TCP(POSS) and PCL/TCP(POSS)-foam, and each scaffold was seeded with approximately $1.8 \times 10^5$ rat BMSCs according to the protocol described in the materials and methods of chapter 4.

6.2.5 Morphology of the cell-scaffolds constructs

SEM was used to assess cell morphology and attachment \textit{in vitro} as described in the materials and methods of chapter 4.
6.2.6 PicoGreen® assay

Proliferation of rat BMSCs in cell-scaffold constructs were studied using PicoGreen® assay as per manufacturer’s protocol (Molecular Probes, PicoGreen dsDNA Quantitation kit) was described in the materials and methods of chapter 4.

6.2.7 Alkaline phosphate (ALP) activity

The functional activity of the proliferated cells was examined by measuring the ALP activity expressed by the cells as described in the materials and methods of chapter 4. Five samples were collected for each time point from each group to ensure sufficient quantity of proteins for the assays.

6.2.8 Statistical analysis

All the data presented are expressed as mean ± standard deviation. An unpaired student’s t-test was used to test the significance level of the data. Differences were considered statistically significant at p<0.05.

6.3 Results and discussions

6.3.1 Fabrication of PCL/TCP(POSS) scaffolds by SES

In order to prepare additive manufactured PCL/TCP scaffolds with adequate mechanical properties for cancellous bone tissue engineering, POSS was used to modify PCL/TCP composite as described in chapter 4. The prepared PCL/TCP(POSS) composite was used to fabricate scaffolds through an in-house screw extrusion system (SES). The fabricated scaffolds were 100% interconnected with pore size of 750-950 μm
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and overall porosity of 65%. The compressive modulus and compressive strength of the fabricated PCL/TCP(POSS) scaffolds are 93.8 MPa and 6.02 MPa, respectively which fall within the range of mechanical properties of cancellous bone (50 – 500 MPa for compressive modulus and 3 – 12 MPa for compressive strength). Hence, PCL/TCP(POSS) scaffolds are suitable to be used for cancellous bone tissue engineering application. Moreover, on the surface of the PCL/TCP(POSS) scaffolds, exposed TCP was noticed, which eventually improves the biomimetic properties of the PCL/TCP(POSS) scaffolds compared to PCL/TCP scaffolds.

6.3.2 Hierarchical PCL/TCP(POSS)-foam scaffolds to improve the functional performance of additive manufactured scaffolds

PCL/TCP(POSS)-foam scaffolds were successfully produced by integrating two scaffold fabrication techniques: additive manufacturing and freeze drying technology. In this hierarchical structured scaffolds, additive manufactured PCL/TCP(POSS) structure provides adequate mechanical properties to be used in cancellous bone tissue engineering application. On the other hand, the porous gelatin foam structure created by freeze drying the gelatin solution acts as cell entrapment system during cell seeding, and thereby improves the functional performance of the additive manufactured scaffolds.

To facilitate the attachment between gelatin and the PCL/TCP(POSS) scaffolds, carboxyl group was introduced onto the surface of PCL/TCP(POSS) scaffolds by treating with NaOH and CH$_3$COOH, respectively. Porous gelatin foam structure with different morphologies were created within the pores of the additive manufactured PCL/TCP(POSS) scaffolds by varying concentration of gelatin solution (Figure 6.1 and Figure 6.2). Porous gelatin foam structures formed at low concentrations of 0.1% and
0.2% (w/v) gelatin solution look unstable. The porous structure starts showing a definite pattern first at concentration of 0.3% (w/v). With the increase of gelatin concentration the pore size of the formed porous gelatin structure becomes smaller (Figure 6.2), and overall the structure becomes more packed (Figure 6.1). From the different groups of gelatin concentrations, porous gelatin foam structure with a concentration of 0.4% (w/v) gelatin has been chosen for further studies, assuming that the structure will entrapment cell, and at the same time as it is not densely packed it will not hinder nutrient flow within the pores of the scaffolds. This group will be denoted just as PCL/TCP(POSS)-foam scaffolds from now on.

Figure 6.1 SEM images of overall PCL/TCP(POSS)-foam scaffolds with porous foam structure created with different concentrations of gelatin solution. The concentration of gelatin used was (a) 0.1% (w/v), (b) 0.2% (w/v), (c) 0.3% (w/v), (d) 0.4% (w/v), (e) 0.5% (w/v) and (f) 0.6% (w/v).
Figure 6.2 SEM images at higher magnification of PCL/TCP(POSS)-foam scaffolds showing morphology of porous foam structure with different concentrations of gelatin solution. The concentration of gelatin used was (a) 0.1% (w/v), (b) 0.2% (w/v), (c) 0.3% (w/v), (d) 0.4% (w/v), (e) 0.5% (w/v) and (f) 0.6% (w/v).

As gelatin is water soluble the stability of the formed porous gelatin foam structure was improved by using cross-linking. Gelatin can be cross-linked using several physical and chemical cross-linking approaches [191-193]. Physical methods which include de-hydrothermal treatment and UV-irradiation are considered less efficient due to less control on the degree of crosslinking. Chemical crosslinking provides better control over the degree of crosslinking, and can be divided into non zero-length and zero-length cross-linkers. However, non-zero length crosslinkers may have an adverse effect in the body upon biodegradation of the hydrogel [192]. In this aspect zero length crosslinkers can be considered as they can facilitate crosslinking without incorporation of foreign structures into the network by activating carboxylic acid residues to react with free amine, resulting in the formation of an amide bond. In this experiment PCL/TCP(POSS)-foam scaffolds were successfully cross-linked using a zero-length EDC/NHS agent based on the experimentally determined conditions [Figure 6.3]. The photograph image of PCL/TCP(POSS) and cross-linked PCL/TCP(POSS)-foam scaffolds has been shown in Figure 6.4.
Figure 6.3 SEM images of PCL/TCP(POSS)-foam scaffolds (a) before and (b) after cross-linking using EDC and NHS.

Figure 6.4 Photograph image of PCL/TCP(POSS)-foam scaffold (left) and PCL/TCP(POSS) scaffold (right).

6.3.3 In vitro cell response

In this study, the proliferative and osteoconductive properties of the hierarchical scaffolds are evaluated by observing rat BMSCs morphology, proliferation and ALP expression. Figure 6.5 shows the SEM images of rat BMSCs cultured on PCL/TCP(POSS) and PCL/TCP(POSS)-foam scaffolds at day 14 and 21. For both of the time points higher levels of cells and cell-sheet accumulation were observed on PCL/TCP(POSS)-foam scaffolds compared to PCL/TCP(POSS) scaffolds. At day 14, cells on PCL/TCP(POSS) scaffolds showed good adherence to the scaffolds surface; on the other hand, the cells on PCL/TCP(POSS)-foam scaffolds showed not only good adherence but also a clear indication of cell-sheet accumulation. At day 21, the accumulation of cell-sheet on PCL/TCP(POSS)-foam scaffolds compared to PCL/TCP(POSS) scaffolds is more distinctive and prominent. Figure 6.6 shows the proliferation of BMSCs in the cell-scaffold constructs studied by PicoGreen® assay. At all the time points PCL/TCP(POSS)-foam scaffolds showed significantly higher amount
of DNA in comparison to PCL/TCP(POSS) scaffolds. PCL/TCP(POSS)-foam scaffolds exhibit 2.6 and 3.5 times higher amount of DNA concentration compared to PCL/TCP(POSS) scaffolds at day 14 and day 3, respectively.

The differentiation behaviour of cells on the scaffolds was evaluated by measuring the ALP activity of the cells on the scaffolds. ALP activity was measured as its study is straight forward and long been recognized [194]. As can be seen from Figure 6.7, the ALP activity of the cells on PCL/TCP(POSS)-foam scaffolds is significantly higher compared to that of PCL/TCP(POSS) scaffolds at all the time points. At day 14 and 28, the ALP activity of PCL/TCP(POSS)-foam scaffolds is 6.3 and 4.9 times higher compared to that of PCL/TCP(POSS) scaffolds, respectively.

These in vitro results suggest that the porous gelatin foam structure significantly improved the functional performance of the extrusion based AM scaffolds for bone tissue engineering.

Figure 6.5 SEM images of cell-scaffold construct of PCL/TCP(POSS) and PCL/TCP(POSS)-foam scaffolds at day 14 and day 21.
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Figure 6.6 PicoGreen® DNA quantification results of rat BMSCs cultured on PCL/TCP(POSS) and PCL/TCP(POSS)-foam scaffolds (*p<0.05).

Figure 6.7 Alkaline phosphate expression normalized to protein content of rat BMSC seeded PCL/TCP(POSS) and PCL/TCP(POSS)-foam scaffolds (*p<0.05).
6.4 Conclusions

A novel PCL/TCP(POSS)-foam scaffold that combines the advantageous properties of AM scaffolds and porous foam scaffolds was developed. The successful integration of extrusion based AM and freeze drying technologies provides a platform to improve the functional performance of the AM scaffolds by providing a cell entrapment system. In the hierarchical structure of PCL/TCP(POSS)-foam scaffolds the macro-sized PCL/TCP(POSS) filaments provide adequate mechanical support for cancellous bone tissue engineering application. On the other hand the porous gelatin foam structure acts as a cell entrapment system, and thereby improves the functional performance of the AM scaffolds. The \textit{in vitro} SEM results showed notably higher level of cells and cell-sheet accumulation on PCL/TCP(POSS)-foam scaffolds compared to PCL/TCP(POSS) scaffolds. The proliferation of BMSCs on PCL/TCP(POSS)-foam scaffolds was significantly higher compared to PCL/TCP(POSS) scaffolds. ALP activity was 5 times higher for PCL/TCP(POSS)-foam scaffolds compared to PCL/TCP(POSS) scaffolds at day 28. These \textit{in vitro} results clearly showed the potential of PCL/TCP(POSS)-foam scaffolds for bone tissue engineering. Furthermore, the porous gelatin foam structure within the filaments of the scaffolds could also be used to supply specific gene and/or different biological factors, as gelatin has the potential to deliver different biological cues [188, 195, 196]. These hierarchical scaffolds could also be used to create different multifunctional 3D matrices that will improve the current status of tissue engineering applications [16].
Chapter 7

*In Vivo Evaluation of Apatite Coated Additive Manufactured Scaffolds in a Rat Calvarial Defect Model*

7.1 Introduction

Development of appropriate synthetic bone graft or scaffold is becoming a key research topic in bone tissue engineering due the added advantages of synthetic bone graft over allograft and/or autogenous cancellous bone graft [3, 6, 123]. In this scaffold based bone tissue engineering approach, the scaffold acts as a temporary matrix to maintain, restore and improve the function of the damaged tissue by mimicking the mechanical and biochemical properties of the native tissue.

To improve the mechanical and biochemical properties of the polymeric scaffolds pristine calcium phosphate ceramic particles have been mixed into the polymer directly [11, 12, 14, 128]. However, an important aspect which has been neglected in the context of bone tissue engineering is the interfacial properties between the ceramic and matrix phases [18], and therefore, limited improvement has been noticed on mechanical properties of polymer/ceramic composite scaffolds compared to polymeric scaffolds [15]. On the other hand, the masking of the ceramic particles by polymer during the blending process diminishes the proliferative and osteoconductive properties offered by ceramic particles [150]. Hence, to improve the proliferative and osteoconductive properties of polymer/ceramic composite scaffolds, mineralizing the scaffolds with a layer of apatite coating is considered an efficient approach. Comprehensive *in vitro* studies have been demonstrated the positive influence of biomimetic apatite coating on
the proliferative and osteoconductive properties of the synthetic scaffolds [128]. However, there are limited in vivo studies which justify the relevancy of biomimetic apatite coating on synthetic scaffolds. It should be noted here that clinically demineralized autograft and allograft bone is more useful than mineralized graft [197-204]. Although, this is not necessarily the case when considering the clinical relevance of mineralization by biomimetic apatite coating on synthetic scaffolds, certainly more in vivo studies are needed to verify the clinical need of mineralization on synthetic scaffolds.

In these contexts, to improve the mechanical properties of the polymer/ceramic composite scaffolds we have developed silanized poly-caprolactone/tricalcium phosphate (PCL/TCP(Si)) scaffolds, which has significantly improved mechanical properties compared to benchmark PCL/TCP scaffolds as described in chapter 4. Moreover, to improve the osteoconductive properties of the PCL/TCP(Si) scaffolds biomimetic coating was given on it as also described in chapter 5. The developed biomimetic apatite coated PCL/TCP(Si) showed excellent mechanical properties and promising proliferative and osteoconductive properties in vitro, which eventually encourages us to initiate further in vivo study.

Extrusion based AM technology fabricates scaffolds with honeycomb structure by laying down the screw extruded filaments in different directions [55]. The effect of different laydown patterns on this type of scaffolds has been conducted in terms of mechanical and in vitro characterization [28, 55, 205]; however, the effect of different lay down pattern in vivo has not been done yet.

To address these issues, in the present study, we investigated the use of high performance extrusion based additive manufactured PCL/TCP(Si) scaffolds coated with
biomimetic apatite coating as an approach for optimizing the bone regenerative capabilities in the critical sized calvarial defect in rat model. We hypothesized that the scaffolds would provide a suitable substrate for cell proliferation and osteogenic differentiation in vivo. In conjunction with the two types of apatite coated scaffolds, namely carbonated hydroxyapatite (CHA) coated scaffolds and CHA-gelatin composite coated scaffolds, the effect of different laydown patterns of the scaffolds have also been investigated.

7.2 Materials and methods

7.2.1 Materials

Poly(ε-caprolactone) (PCL) (Mₖ: 80,000), 3-glycidoxypropyl trimethoxysilane (GPTMS), gelatin (type A, from porcine skin), acetic acid (CH₃COOH), calcium chloride (CaCl₂), potassium hydrophosphate (K₂HPO₄), phosphoric acid (H₃PO₄, 85% solution in water), sodium carbonate (Na₂CO₃) and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich, Singapore. Tri-calcium phosphate (TCP) was purchased from Progentix, The Netherlands.

7.2.2 PCL/TCP(Si) composite preparation and scaffolds fabrication

PCL/TCP(Si) composite were synthesized, and subsequently PCL/TCP(Si) scaffolds were fabricated according to the process described in the materials and methods of chapter 4. In this experiment the scaffolds were fabricated with two different laydown pattern namely 0°/90° and 0°/60°/120° to study whether there is any effect of laydown pattern in vivo. The size of the scaffolds was 5 mm in diameter and 2 mm in thickness.
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7.2.3 Biomimetic CHA and CHA-gelatin coating on PCL/TCP(Si) scaffolds

The PCL/TCP(Si) scaffolds were coated with CHA and CHA-gelatin coating according to the process described in chapter 5.

7.2.4 In vitro studies

7.2.4.1 Cell seeding on scaffolds

Four groups of scaffolds were prepared for in vitro study namely, PCL/TCP, PCL/TCP(Si), PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin. The reason for including PCL/TCP scaffolds is just for a comparative evaluation with PCL/TCP(Si) scaffolds with rat BMSCs. Each scaffold was seeded with approximately $1.8 \times 10^5$ rat BMSCs according to the protocol described in the materials and methods of chapter 4.

7.2.4.2 Morphology and viability study of the cell-scaffold constructs

SEM and confocal imaging were used to assess cell morphology, viability and attachment in vitro as described in the materials and methods of chapter 4.

7.2.4.3 ALP

The functional activity of the proliferated cells was examined by measuring the ALP activity expressed by the cells as described in the materials and methods of chapter 4. Five samples were collected for each time point from each group to ensure sufficient quantity of proteins for the assays.
7.2.4.4 *PicoGreen® Assay*

Proliferation of rat BMSCs in cell-scaffold constructs were studied using PicoGreen® assay as per manufacturer’s protocol (Molecular Probes, PicoGreen dsDNA Quantitation kit) was described in the materials and methods of chapter 4.

7.2.5 *In vivo studies*

7.2.5.1 *Animal Surgery*

Twenty one skeletally-matured male Lewis rats were obtained from Animal Resources Centre, Canning Vale, WA. The rats were housed at the QUT Medical Engineering Research Facility (MERF) at the Prince Charles Hospital, Chermside. The animals received water and pelleted ration ad libitum throughout the experiment. The animal surgery and related *in vivo* characterizations were carried out by Prof Dietmar Hutmacher’s group at Institute of Health and Biomedical Innovation, Queensland University of Technology, Australia.

7.2.5.2 *Experimental design*

The rats were subjected to critical sized bone defects creation in their skull and implantation of PCL/TCP(Si) scaffolds coated with CHA and CHA-gelatin coating. Accordingly, the rats were assigned to four groups of six, as follows:

Positive Control group: implantation of PCL/TCP(Si) scaffold (without surface modification)

Negative Control group: the calvarial bone defects will be left empty.

Treatment group I: implantation of PCL/TCP(Si)-CHA scaffolds.
7.2.5.3 Anesthesia and Pre-operative considerations

All rats were operated under general anaesthesia. Buprenorphine (0.01 - 0.05, subcutaneously) were used pre-operatively for preemptive analgesia and post-operatively every 6-12 hour as pain killer. General anaesthesia was provided by using mixture of ketamine and xylazine (75-100 Ket + 5-10 Xyl, intraperitoneally in same syringe). Rats were handled briefly by hand for intraperitoneal injection of anaesthetic and then released into a separate cage until they become unconscious and ready for surgery. During anaesthesia, surgery and immediate post-operative period, the rats will were kept on a heating pad and after that, they were transferred to a clean warmed cage for recovery.

The front parietal region was prepared by clipping hair with a delicate clipper and vigorous disinfection was achieved by application of chlorhexidine in alcohol solution. One dose of broad-spectrum antibiotic was given to the rats, immediately before surgery as for prophylaxis.

7.2.5.4 Surgical procedure

In order to produce critical size bone defects, a sagittal incision of approximately 20 mm was performed over the scalp of the animal. A full-thickness bone defect (5 mm in diameter) was trephined in the centre of each parietal bone (two defects per calvarium) using a slow speed dental drill with irrigation to prevent heat damage of the host bone. While drilling down the bone, caution was taken not to damage the underlying exposed
*dura mater* at the bottom of the defect. A strip 2-mm wide of the marginal periosteum surrounding the defect was then removed. According to the implantation plan, both bone defects in each rat were implanted with one of the treatment modalities described above. Incisions were sutured and animals were allowed to recover for 12 weeks of postsurgery, after which they were sacrificed by CO₂ inhalation. To collect the implants, the skin was dissected, and the defect sites were removed along with the surrounding bone. The biopsied specimens were fixed and prepared for microCT analysis and mechanical testing.

7.2.5.5 MicroCT

At 3 months after surgery, *in vivo* mineralization within the constructs was quantified using a Micro-CT 40 scanner (Scanco Medical, Brüttisellen, Switzerland) at a voxel size of 36 μm. Samples were evaluated at a threshold of 220, a filter width of 0.8 and filter support of 1.0. The data were volumetrically reconstructed using the reconstruction software from scanco. From the three-dimensional image, a cylindrical region of interest (ROI) of defect size of 5 mm diameter was selected for analysis. This ROI corresponds to the original defect location. X-ray attenuation was correlated to sample density using a standard curve generated by scanning hydroxyapatite phantoms with known mineral density. Mineralized matrix volume or bone volume fraction, and mineral density were quantified throughout the entire construct and presented as bone volume in mm³.

7.2.5.6 Mechanical testing

After sacrifice, the rat calvaria were retrieved, wrapped in wet gauze and stored at -20 °C until analysis. Upon thawing, the calvaria were potted in a Petri dish with
polymethylmethacrylate (Meliodent Rapid Repair, Heraeus Kulzer) to enable stable fixation for the mechanical testing. Non-destructive micro-compression on the calvaria defects was performed using a Micro Tester 5848 (Instron) with a 10-N load cell. An indenter probe of 0.5 mm diameter was micro-fabricated for the test. Micro-compressions of up to 50% strain were conducted at an average of eight different locations on each defect site, and the load-displacement and stiffness (compression modulus) were determined. The probe locations were identified to be the pore spaces (between the scaffold struts) of the constructs so as to measure the modulus of regenerated tissue rather than scaffold material. Push-out tests were conducted to evaluate the functional mechanical integration of the tissue-engineered constructs into the host calvaria, and were performed on the Micro Tester 5848 (Instron) with a 1-kN load cell [206, 207]. An indenter probe of 4.5 mm diameter, slightly smaller than the scaffold diameter of 5 mm, was fabricated for the test. After the micro-compression tests were completed, the calvarial specimens were tested to failure and the final yield was determined. Four to eight specimens were used for each group. Intact rat calvaria were also used as controls.

7.2.6 Statistical analysis

Statistical analysis was performed for all the quantitative results using Student’s t-test for comparing means from two independent sample groups. A confidence level of 95% was used (p< 0.05).
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7.3 Results and discussions

7.3.1 PCL/TCP(Si) composite preparation, scaffolds fabrication and biomimetic coating on scaffolds

To improve the mechanical properties of the additive manufactured PCL/TCP scaffolds, interfacial bonding between PCL and TCP was enhanced by modifying TCP using GPTMS as a coupling agent. PCL/TCP(Si) scaffolds were fabricated from the synthesized PCL/TCP(Si) composite using an in house SES. In this study, the fabricated scaffolds were 100% interconnected with porosity of 67% and 71% for 0º/60º/120º and 0º/90º patterned scaffolds, respectively.

On the other hand, in order to improve the osteoconductive property of PCL/TCP(Si) scaffolds, biomimetic CHA and CHA-gelatin were coated onto PCL/TCP(Si) scaffolds through a facile biomimetic process. The detailed mechanism and characterization of the PCL/TCP(Si), PCL/TCP(Si)-CHA and PCL/TCP(Si)-gelatin scaffolds have been discussed in the results and discussion section of chapter 5.

7.3.2 In vitro studies

In this study, the proliferative and osteoconductive properties of the prepared scaffolds are evaluated by observing rat BMSCs morphology, proliferation, and ALP expression. In the in vitro studies the objective was to evaluate the coated scaffolds. Scaffolds with different laydown pattern were not tested in vitro, as no difference has been noticed from the previous study of the senior author’s group [55].

Figure 7.1 shows the confocal laser microscopy image of rBMSCs cultured on PCL/TCP, PCL/TCP(Si), PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin scaffolds.
at different culture time. At both day 7 and day 14, BMSCs stretched well over the struts and distributed in PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin scaffolds, while qualitatively fewer BMSCs were observed in non-coated PCL/TCP and PCL/TCP(Si) scaffolds.

Figure 7.2 shows the proliferation of rBMSCs in the cell-scaffold constructs studied by PicoGreen® assay. At all the time points, both the CHA coated and CHA-gelatin composite coated PCL/TCP(Si) scaffolds exhibit higher amount of DNA in comparison to non-coated PCL/TCP and PCL/TCP(Si) scaffolds. At all the time points PCL/TCP(Si)-CHA-gelatin scaffolds exhibit significantly higher amount of DNA in comparison to non-coated PCL/TCP(Si) scaffolds. At day 14, PCL/TCP(Si)-CHA scaffolds exhibit higher amount of DNA in comparison to non-coated PCL/TCP and PCL/TCP(Si) scaffolds, whereas PCL/TCP(Si)-CHA scaffolds exhibit higher amount of DNA compared to PCL/TCP(Si) scaffolds.

<table>
<thead>
<tr>
<th></th>
<th>PCL/TCP</th>
<th>PCL/TCP(Si)</th>
<th>PCL/TCP(Si)-CHA</th>
<th>PCL/TCP(Si)-CHA-gelatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>Day 14</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 7.1 Confocal laser microscopy with depth projection images reconstructed from multiple horizontal images shows 3D distribution of cells within the scaffolds of PCL/TCP, PCL/TCP(Si), PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin.
Figure 7.2 PicoGreen® DNA quantification results of BMSCs cultured on PCL/TCP, PCL/TCP(Si), PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin scaffolds (*p<0.05).

ALP is a biomarker of osteogenic differentiation; hence its expression indicates differentiation of rBMSCs down the osteoblast lineage. As can be seen from Figure 7.3, all the scaffolds demonstrated a steady growth in ALP activity. For all the time points PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin showed higher level of ALP activity compared to PCL/TCP and PCL/TCP(Si) scaffolds. It should also be noted here that no difference between CHA coating and CHA-gelatin composite coating have been noticed.

From the in vitro results it can be said that CHA coated and CHA-gelatin composite coated scaffolds have greater potential compared to non-coated scaffolds. In the following section in vivo effect of the coatings will be discussed. Moreover, different patterns of scaffolds were evaluated to identify the influence of different lay down pattern.
Figure 7.3 Alkaline phosphatase expression normalized to protein content of rMSC cultured on PCL/TCP, PCL/TCP(Si), PCL/TCP(Si)-CHA and PCL/TCP(Si)-CHA-gelatin scaffolds.

7.3.3 In vivo studies

7.3.3.1 Animal surgery

Critical size bone defects were created in the scull, and the scaffolds were implanted inside the scaffolds as shown in Figure 7.4. After 12 weeks, the animals of all groups were euthanized and the specimens were removed along with surrounding bone for further evaluations. No wound infection appeared during the experiment, and all the animals were well.
Figure 7.4 The scull defects were drilled with a 5 mm dental drill (A), the bone was carefully removed without damaging the dura (B) and the scaffolds were fitted in (C).

7.3.3.2 Micro-CT

The bone regeneration capacity of scaffolds with different surface modifications was assessed in vivo after transplantation in a rat calvarian defect. Transplants were recovered after 12 weeks, subjected to micro-CT analysis and then processed to mechanical testing. Micro-CT analysis showed a significant higher degree of newly formed bone matrix in all experimental groups compared to the negative control group (empty defect) (Figure 7.5). However, no significant difference could be found with regard to the mineral density of newly formed bone matrix among the coated and non-coated PCL/TCP(Si) scaffolds (Figure 7.6). Though in vitro we have found significant difference between coated and non-coated PCL/TCP(Si) scaffolds, while studying in vivo no significant difference have been noticed. The difference in findings between in vitro and in vivo studies once again reminds us that, just the in vitro study is not sufficient to predict the clinical outcome [208].

However, all groups of scaffolds with a 0º/90º configuration of the fibers showed a tendency of more bone formation compared to the scaffolds with a 0º/60º/120º configuration of the fibers. This result can be correlated with the porosity of the 0º/90º
and 0°/60°/120° as it is known that in vivo higher porosity result in greater bone growth [20]. In this study the scaffolds with 0°/90° pattern have 71% porosity, whereas the scaffolds with 0°/60°/120° pattern have 67% porosity (Figure 7.7).

![Figure 7.5](image)

Figure 7.5 The PCL/TCP-0°/90° Scaffold (A) showed more newly formed bone matrix compared to PCL/TCP-0°/60°/120° Scaffold (B). The PCL/TCP-CHA-0°/90°/120° Scaffold showed the maximum bone formation among all the groups (C), without significant differences. Picture D, E and F showed the bone formation of the PCL/TCP-CHA-0°/60°/120°, PCL/TCP-CHA-Gel-0°/90° Scaffold and PCL/TCP-CHA-Gel-0°/60° Scaffold, respectively. The negative control (empty group) showed no newly formed bone matrix (G).

![Figure 7.6](image)

Figure 7.6 All the experimental groups showed significantly more newly formed bone matrix compared to the empty defect. No significant differences could be detected between the experimental groups. (*P < 0.05).
7.3.3.3 Mechanical testing

To test the mechanical integrity of the newly formed tissue within the scaffold pore space, micro-compression was performed on harvested calvaria at 8 different locations within pore spaces of each scaffold (Figure 7.8).

All defects, treated with PCL/TCP(Si)-Scaffolds with a 0°/90° configuration of the fibers showed a higher stiffness and compared to the PCL/TCP(Si)-0/60°-Scaffolds (Figure 7.9). The tissue stiffness of the PCL/TCP-(si)-0/90° and the PCL/TCP-(si)-CHA-0/90° were comparable to that of intact bone (Figure 7.9A). The defects treated with PCL/TCP-(si)-CHA-gelatin-0/60° and 0/90°-Scaffolds showed significant lower stiffness compared to all the other experimental groups. The push-out tests were performed to evaluate the mechanical integration of the constructs with the host calvaria (Figure 7.9B), an important aspect from acraniofacial treatment point of view. After 12 weeks, the defects treated with PCL/TCP-(si)-0/90° Scaffolds, showed a similar push-out strength as that of host bone tissue, suggesting new bone was forming from the periphery of the defect edges and encouraging integration of the implant within the defect site (Figure 7.9B). The PCL-TCP(Si) scaffolds with the 0°/90° configuration of the fibers showed again a higher push-out strength compared to the 0°/60°/120° scaffolds.
Figure 7.7 Surface analysis of PCL/TCP(Si) scaffolds with lay down pattern of 0°/60°/120° and 0°/90° showing significant difference in (a) porosity, (b) trabecular space, (c) scaffolds volume and (d) scaffolds surface.

Figure 7.8 A sample calvaria 12 weeks (A) is undergoing a microcompression test. A biopsy punch was used to push-out scaffolds to evaluate load of fracture and mechanical integration with the host calvaria (B). Micro-compression was performed within pore spaces of the scaffolds as well as within the empty defect (Empty), on host calvarial bone (Bone), implant struts (Strut), and non-bony tissue as controls (Tissue). Additionally, push-out tests were performed on host calvarial bone as controls.
Figure 7.9 Micro-compression tests and push out tests were performed after 12 weeks. Stiffness (A) and Load of fracture (B) are reported here. Regenerated tissue within the PCL/TCP-(si)-0/90°-Scaffold group and the mPCL/TCP-(si)-CHA-0/90°- Scaffold group showed superior stiffness and the highest push-out strength of all experimental groups. Significant values are represented as *P < 0.05.
7.4 Conclusions

In this study additive manufactured PCL/TCP(Si) scaffolds coated with apatite were evaluated *in vivo*. The surface coated scaffolds showed excellent results *in vitro* but not significantly distinct results in vivo, which eventually highlight the complexity in translating *in vitro* findings into the clinical arena and the undoubted importance of *in vivo* study. Along with the effect of surface modification, the effect of the lay down pattern of the scaffolds’ was also evaluated. For each experimental group of coated and non-coated scaffolds, the fibre configuration was 0º/90º and 0º/60º/120º. To our knowledge, no other group reported any study in bone regeneration, regarding the alignment of the struts. The experimental groups with the 0º/90º fibres alignment showed a significantly higher bone regeneration potential compared to the 0º/60º scaffold group, in respect of the newly formed bone matrix, the stiffness of the bone matrix and the maximum load of fracture. In the 0º/90º scaffold group, a better incorporation of cells from the host, as well as a better supply of nutrients might be reasons for this improved bone regeneration. This study demonstrated that a surface modification of PCL/TCP(Si) scaffolds can influence their regenerative potential for bone tissue engineering applications. Beside the surface modification, the alignment of the scaffold fibres influences the bone regeneration potential of the scaffolds. A 0º/90º-alignment of the scaffold fibres showed a better regeneration of bone tissue.
Chapter 8

Conclusions and Recommendations

8.1 Conclusions

This research was initiated with the development of polymer/ceramic composites with improved interfacial interaction by the use of different coupling agents. Additive manufactured (AM) scaffolds were fabricated with the developed polymer/ceramic composite using an in-house screw extrusion system (SES), and subsequently characterized. The developed scaffolds were further modified to improve the osteoconductive properties and cell entrapment efficiency. The scaffolds were characterized through different physical characterizations, and as well as in-vitro characterizations. The conclusions and significance of this research project are summarized under the following headings:

- Improvement of the mechanical properties of the additive manufactured scaffolds.
- Improvement of the proliferative and osteoconductive properties of the additive manufactured scaffolds.
- Improvement of the functional performance of the additive manufactured scaffolds.
- Evaluation of the biomimetic composite coated additive manufactured scaffolds in vivo.
8.1.1 Improvement of the mechanical properties of the additive manufactured scaffolds

To improve the mechanical properties of the scaffolds two different coupling agents, namely silane and POSS have been used in this research project. The main idea of using a coupling agent was to improve the interfacial interaction between the ceramic and polymer phase.

The developed silanized PCL/TCP scaffolds showed 6 times higher compressive modulus and 2.3 times higher compressive strength than those of PCL/TCP scaffolds; and the values fall within the lower range values of cancellous bone. *In vitro* confocal images showed homogenous BMSCs distribution on GPTMS modified PCL/TCP scaffolds. PicoGreen® data showed that proliferation rate of BMSCs on modified PCL/TCP scaffolds was equally as good as unmodified PCL/TCP scaffolds. The overall *in vitro* BMSCs culture signifies that there is no detrimental effect of GPTMS on cells’ viability and proliferation.

The POSS modified PCL/TCP scaffolds showed 6.8 times higher compressive modulus and 3.4 times higher compressive strength than those of PCL/TCP scaffolds. Notably the compressive modulus and compressive strength of the native cancellous bone are 93.8 MPa and 6 MPa, respectively, and thus mechanical properties of POSS modified PCL/TCP scaffolds falls within the lower range of those of cancellous bone. Moreover, the SEM observation of POSS modified PCL/TCP scaffolds showed exposed TCP particles on the surface of the scaffolds. The exposed TCP was verified by XPS. This result is also significant, since a common problem in polymer/ceramic scaffolds is the covering up of ceramic particles by polymer matrix. The exposed TCP on the POSS
modified PCL/TCP scaffolds help to improve the proliferative capability of the scaffolds as shown by SEM and confocal images.

The use of coupling agent to improve the interfacial interaction between the ceramic and polymer phase has long been ignored in the development process of composite material for bone TE scaffolds. Hence, this study is remarkably significant as it shows a new perspective to modify scaffolds for better mechanical properties.

8.1.2 Improvement of the proliferative and osteoconductive properties of the additive manufactured scaffolds

In order to improve the proliferative and osteoconductive properties, PCL/TCP(Si) scaffolds were coated with carbonated hydroxyapatite (CHA)-gelatin composite by biomimetic co-precipitation process. The successful incorporation of CHA-gelatin composite coating was verified by SEM and XPS analysis. It should be noted that cells attached to and growing on the bars of additive manufactured scaffolds experience different mechanical constraints in comparison with other types of scaffolds. Coatings on the bars of the additive manufactured scaffolds experience tensile forces due to the free tissue bridges and thus flaking of the coating layer occurs. In this aspect a thin layer of composite coating is desirable. The thickness of developed CHA-gelatin composite coating was 650 nm and developed thin coating did not show any flaking phenomenon on the bars of the additive manufactured PCL/TCP(Si) scaffolds during BMSCs culture. SEM and confocal images demonstrated notably high levels of cells and cell-sheet accumulation with uniform distribution on PCL/TCP(Si)-CHA-gelatin scaffolds compared to PCL/TCP(Si) scaffolds. The proliferation rate of BMSCs on PCL/TCP(Si)-CHA-gelatin scaffolds was 2.0 times higher than that of PCL/TCP(Si) scaffolds,
respectively, by day 10. *In vitro* RT-PCR and western blotting testing showed higher level of osteoconductive property on PCL/TCP(Si)-CHA-gelatin compared to PCL/TCP(Si) scaffolds. Thus, this study is quite significant as it provides a new perspective to the potential of biomimetic composite coating on additive manufactured scaffolds.

### 8.1.3 Improvement of the functional performance of the additive manufactured scaffolds

To improve the functional performance of the AM scaffolds by providing a cell entrapment system, a novel PCL/TCP(POSS)-foam scaffold that combines the advantageous properties of AM scaffold and porous foam scaffold was developed. In the hierarchical structure of PCL/TCP(POSS)-foam scaffolds the macro-sized PCL/TCP(POSS) filaments provide mechanical support and the porous gelatin foam structure acts as a cell entrapment system. From the manufacturing point of view, to fabricate hierarchical scaffolds, our developed approach is considerably simpler than combining electrospinning with AM. *In vitro* SEM results showed notably higher level of cells and cell-sheet accumulation on PCL/TCP(POSS)-foam scaffolds compared to PCL/TCP(POSS) scaffolds. The proliferation of BMSCs on PCL/TCP(POSS)-foam scaffolds was also significantly higher compared to PCL/TCP(POSS) scaffolds. ALP activity was 5 times higher for PCL/TCP(POSS)-foam scaffolds compared to PCL/TCP(POSS) scaffolds at day 28. These *in vitro* results clearly showed the potential of PCL/TCP(POSS)-foam scaffolds for bone tissue engineering. From all the findings it can be claimed that this study provides an efficient alternative to fabricate hierarchical scaffolds with improved functional performance for bone tissue engineering.
8.1.4 Evaluation of the biomimetic composite coated additive manufactured scaffolds in vivo

The study is a collaborative work with Prof Dietmar Hutmacher’s group at Institute of Health and Biomedical Innovation, Queensland University of Technology (QUT), Australia. The scaffolds fabrication and in vitro characterizations were done at NUS, whereas the animal surgery and in vivo characterizations were done at QUT. This study evaluates the bone regeneration capabilities of additive manufactured PCL/TCP(Si) scaffolds coated with biomimetic apatite coating in the critical sized calvarial defect of an animal model. Moreover, two different lay down patterns, namely, 0°/90° and 0°/60°/120° of the scaffolds were also investigated in the animal model. The results showed that a surface modification of PCL/TCP(Si) scaffolds can influence their regenerative potential for bone tissue engineering applications. However, despite the excellent results in vitro, the in vivo performance of the coated scaffolds was not significantly higher compared to non-coated scaffolds, which eventually highlight the complexity in translating in vitro findings into the clinical arena and justifies the undoubted importance of in vivo study. On the other hand, all the experimental groups with the 0°/90° fibres alignment either coated or non-coated showed a significantly higher bone regeneration potential compared to the 0°/60° scaffold group, with respect of the newly formed bone matrix, the stiffness of the bone matrix and the maximum load of fracture. In the 0°/90° scaffolds group, a better incorporation of cells from the host, as well as a better supply of nutrients might be reasons for better bone regeneration. This study signifies that the alignment of the scaffolds’ fibres influence the bone regeneration potential of the scaffolds in vivo. To our knowledge this is the first reported study on in vivo bone regeneration regarding the alignment of the struts.
8.2 Limitations and recommendations

This research focused on the improvement of mechanical and functional performance of the additive manufactured scaffolds for bone tissue engineering. Though significant results were achieved, future research should address the following limitations:

- This study focused only on PCL based composite, PCL/TCP, and did not explore other composites based on PLGA or PLLA polymer. The reason for this focus on only PCL based composite is acceptable, as it is known that the chemistry which is true for PCL is also true for other aliphatic polyester based polymers like PLGA and PLLA. However, in terms of mechanical properties PLGA and PLLA are superior compared to PCL. Therefore, future studies should attempt to use other mechanically stronger polymers instead of PCL to get the required mechanical properties of both cancellous and cortical bone.

- The successful regeneration of tissue largely depends on how tissue replacing the biodegradable scaffolds. This process is executed via controlled transfer of load or function back to the host tissue as the scaffolds degrade and resorb. Therefore, the study of degradation kinetics of the developed silanized and POSS modified PCL/TCP scaffolds could be next phase of study.

- Apatite coating on the scaffolds can be used to deliver proteins and/or growth factors.

- Porous gelatin foam structure within the filaments of the scaffolds could also be used to supply specific gene and/or different biological factors, as
gelatin has the potential to deliver different biological cues. The hierarchical scaffolds could also be used to create different multifunctional three dimensional matrices that will improve the current status of tissue engineering applications.

- Extensive animal study on these scaffolds is highly recommended.
References:


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List of publications

Journals:
4. M. Tarik Arafat, Xu Li, Ian Gibson. Recent trends and challenges in additive manufactured scaffolds. [To be submitted].

Conferences:
1. M. Tarik Arafat, Christopher X. F. Lam, Wong S. Yee, Chaobin He, Xu Li, Ian Gibson. High performance rapid prototyped scaffolds for bone tissue engineering. MRS Fall meeting 2010. Boston, Massachusetts, USA.
2. M. Tarik Arafat, Christopher X. F. Lam, Andrew K. Ekputra, Xu Li, Ian Gibson. Biomimetic composite coating on poly (ε-caprolactone) and silane modified tricalcium phosphate scaffolds for bone tissue engineering. TERMIS AP 2010 meeting. Sydney, Australia.


Appendix A

Improving the Mechanical Properties in Tissue Engineered Scaffolds

A.1 Introduction

Many patients who are in need of organ transplants suffer greatly and may even die from the lack of suitable donors with the demand for artificial organs far exceeding supply. In addition, bone grafting procedures are routinely carried out on a daily basis. Such bone grafts would not be required should there be satisfactory replacement bones. Scaffold-based tissue engineering aims to aid in the repair and regeneration of bone defects. Using this approach, scaffolds act as platforms that carry cells or therapeutic agents for regenerative therapies. To effectively achieve this, the scaffold should be osteoconductive, osteoinductive, biodegradable, highly porous and should have suitable mechanical properties and surface chemistry. To date, scaffold structures have only been used with some success in low-load bearing applications, despite the large variety of biomaterials and fabrication techniques explored in the last two decades.

Poly (ε-caprolactone) (PCL) is a semicrystalline, bioresorbable polymer belonging to the aliphatic polyester family. Its melting temperature is 60°C [3]. PCL has been used to make three-dimensional porous scaffolds, with a fully interconnected pore network, using Fused Deposition Modeling (FDM). β-Tricalcium phosphate (β-TCP) is a bioactive and biocompatible ceramic that has the ability to bind directly to bones. It has been used widely in orthopedic and dental applications due to its solubility and bone
remodeling capabilities. Composites including PCL-TCP are presently being used extensively as they have the suitable properties of both the polymer and ceramic. Recent studies show the suitability of PCL-20% $\beta$-TCP scaffolds fabricated by FDM for low-load bearing bone tissue engineering applications. In this study, we demonstrate the possibility of increasing the mechanical properties of such scaffolds by introducing a through-hole. A number of scaffolds with through-holes of various sizes were fabricated in order to study the effect of the through-hole diameter on the stiffness of the complete scaffold. As well as possibly improving the mechanical properties of a scaffold, it is conjectured that through-holes may also become useful for the channeling or storage of nutrients.

A.2 Materials and methods

A.2.1 Scaffold processing

A.2.1.1 Scaffold Material:

For experiments we used PCL-20% $\beta$-TCP biocomposite. The composite was prepared by combining the PCL polymer and $\beta$-TCP in methylene chloride (JT Baker). The distribution of $\beta$-TCP within the composite was assessed and ensured to be homogeneous by visual inspection and analyzed using a microcomputed tomography ($\mu$-CT) machine (Skyscan1076, Belgium).
A.2.1.2 Scaffold Fabrication:

In house screw-extrusion system (SES) has been used to fabricate the scaffolds. Once the material is fed into the SES, the material melts to a semi-liquid state. The semi-liquid material is then extruded through the nozzle due to pressure created by screw rotation. The extruded material is deposited in layers in a similar way to the commercial FDM process. The material solidifies and bonds to the preceding layer. After a layer is completed, the height of the extrusion head is increased and the subsequent layers are built to construct the entire scaffold.

The SES was used to fabricate scaffolds with 0/90° lay down pattern and bulk dimension of 30 mm * 30 mm * 7 mm. During the fabrication the processing temperature was set at 85 °C, the filament separation distance (centre to centre horizontal distance between adjacent filaments) was set to 1.5 mm and the nozzle diameter 0.5 mm. After the scaffolds were fabricated, through holes were drilled and subsequently scaffolds were cut into 6 mm * 6 mm * 7 mm dimensions for analysis. The through holes were drilled because this technique was considered to be the simplest method for incorporating a sealed through hole into this type of scaffold. To study the relationship between hole size and strength, drill bits of 1.3 mm, 1.5 mm, 1.8 mm, 2.0 mm, 2.3 mm sizes were used.

A.2.2 Morphology study by SEM

Scaffold morphology and pore size were studied by using Scanning Electron Microscopy (SEM). Scaffolds were gold sputter coated by using a JEOL fine sputter
coater (JFC-1200) for 90 seconds at 10 mA. The SME images were taken by Philips XL30 FEG at a beam intensity of 10 kV.

**A.2.3 Mechanical characterization**

Compression tests were conducted on scaffolds by using an Instron 4502 Uniaxial testing system and 5kN load cell (Canton, MA, USA). From every batch five samples were tested adopting the guidelines for compression testing of acrylic bone cement set in ASTM F451-99a. The scaffolds were compressed at a rate of 1 mm/min up to a strain level of approximately 80%. The stress-strain ($\sigma$-$\varepsilon$) curves were obtained to evaluate the compressive modulus. The modulus was calculated from the stress-strain curve as the slope of the initial linear portion of the curve.

**A.3 Results**

**A.3.1 Scaffold morphology**

Figure 2 shows SEM images of scaffolds with different sizes of drilled hole. It can be seen from the figure that, at the wall of the larger holes, a greater amount of material has been melted by the drill bit during drilling.

**A.3.2 Mechanical properties of the scaffolds**

The stiffness of scaffolds with different hole size is given in Table 1. The effect of the diameter of the holes on the stiffness of the scaffolds is given in Figure 3. Table 1
and figure 3 show an increase of about 37% in stiffness for scaffolds with hole diameter 2.3 mm compared with that of scaffolds without a drilled hole.

Table A-1: Calculated compressive stiffness for the scaffolds with different hole diameter.

<table>
<thead>
<tr>
<th>Diameter of hole (mm)</th>
<th>Stiffness (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without hole</td>
<td>25.86 ± 1.09</td>
</tr>
<tr>
<td>1.3</td>
<td>22.74 ± 1.3</td>
</tr>
<tr>
<td>1.5</td>
<td>27.61 ± 1.14</td>
</tr>
<tr>
<td>1.8</td>
<td>27.65 ± 1.56</td>
</tr>
<tr>
<td>2.0</td>
<td>34.29 ± 0.55</td>
</tr>
<tr>
<td>2.3</td>
<td>35.54 ± 2.64</td>
</tr>
</tbody>
</table>

Fig. A-1: SEM images of the scaffolds.
Table A-1 and Figure A-2 show that for the smaller diameter hole the effect on stiffness is negative. After this we appear to get a threshold value for the hole diameter. Finally, there is noteworthy increment in the stiffness for the larger hole diameter.

The stiffness of the scaffold with hole diameter 1.3mm is lower than that of the scaffolds without hole. For the 1.5 mm hole diameter the difference in stiffness is also not remarkable. Therefore, it can be said that hole diameter smaller than the filament separation distance has negative impact on the stiffness of the scaffolds. This may be because, for a smaller diameter hole, the structure is damaged during the hole making process. Evidence of this can be observed in the developed micro cracks as shown in Figure A-3.

It should be noted that for 1.5 mm hole and for 1.8 mm hole we observed only a 6.9% increment in stiffness. However, a large change in stiffness was found for 2.0mm hole diameter. For 2.0 mm and 2.3 mm hole diameters, the stiffness increases by 32.6% and 37.4% respectively than that of scaffolds without drilled holes. Hence, it can be said
that 1.8mm diameter hole acts as a threshold value for 1.5 mm filament distance and hole diameters larger than the filament distance have significant effect on the stiffness of the scaffold. From Figure A-4, it can be seen that larger diameter drilled holes involve more material in the column making process. As the amount of fused material is more for the larger hole diameter, the strength of the scaffold has increased correspondingly. It can be seen clearly that the holes mask off the interconnected pores in the remainder of the scaffold. Such columns can act as a means for storing nutrients or for channeling flow through the scaffold. The number and distribution of the columns must therefore be limited in relation to the remainder of the scaffold, but nonetheless can be an important contributor to the overall effectiveness of the scaffold for tissue regenerative purposes.

Fig. A-3: Micro cracks at the wall of the hold with 1.5 mm diameter.

Fig. A-4: Molten material acts as a column and increases the strength of the scaffold. This image is for 2.3 mm hole.
A.5 Conclusions

This experiment demonstrated the possibility of increasing the mechanical properties of the PCL-20% β-TCP scaffolds by making a through hole. From the experiment we can conclude that, for a specific filament distance there is a threshold value of the hole diameter. A hole larger than that of the threshold value has a significant impact on increasing the strength of the scaffold. In addition it is believed, this column type hole can also be useful for channeling or storage of nutrients. By using this through hole for nutrient storage we can make scaffolds more suitable for load bearing application without decreasing the effectiveness for tissue regeneration. Future study may investigate how to incorporate through-hole into the direct fabrication process.