Design and validation of a scaffold library and algorithm for tissue engineering applications

May Win Naing

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DESIGN AND VALIDATION OF A SCAFFOLD LIBRARY AND
ALGORITHM FOR TISSUE ENGINEERING APPLICATIONS

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SCHOOL OF MECHANICAL & AEROSPACE ENGINEERING
NANYANG TECHNOLOGICAL UNIVERSITY

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Design and Validation of a Scaffold Library and Algorithm for Tissue Engineering Applications

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School of Mechanical & Aerospace Engineering

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ABSTRACT

A novel CAD system of structures based on convex polyhedral units has been created for use with Rapid Prototyping (RP) technology in tissue engineering applications. The prototype system is named the ***COMPUTER AIDED SYSTEM FOR TISSUE SCAFFOLDS*** or ***CASTS***.

CASTS consists of a basic library of units that can assemble uniform matrices of various shapes. Each open-cellular unit is a unique configuration of linked struts. Together with an algorithm which allows the designer to specify the unit cell and the required dimensions, the system is able to automatically generate a structure that is suitable for the intended tissue engineering application. Altering the parameters can easily change the desired shape and spatial arrangement of the structures.

The main advantage of CASTS is the elimination of reliance on user skills, much unlike conventional techniques of scaffold fabrication. From a small range of basic units, many different scaffolds of controllable architecture and desirable properties can be designed. The system interface of CASTS in Pro/ENGINEER is user friendly and allows complete transfer of knowledge between users without the need for complex user manuals.

A femur implant was successfully fabricated using Selective Laser Sintering (SLS) and a standard commercial material Duraform™ Polyamide. However, it was seen that there was some raw powder trapped inside the concept model and further investigations were carried out to minimise the incidence of trapped powder in the scaffolds. A disc shape scaffold was designed for this purpose. In this disc-shaped design, four different strut

lengths were used, resulting in four different pore sizes and porosity. Scaffolds of the one-unit layer cells built using Duraform™ Polyamide were then examined under a light microscope to check the consistency and reproducibility of the microstructures.

Removing trapped powder was difficult for small pore sizes below 0.500mm (500µm) in diameter. Lowering the part bed temperature of the SLS system was found to be effective in reducing the amount of powder trapped. Ultrasonic cleaning was employed to test if it could remove the powder still trapped after manual cleaning. The results suggested that ultrasonic cleaning might have to be combined with another means of cleaning to increase the rate of success.

Three types of biomaterials were tested on CASTS: PEEK, PEEK-HA biocomposite, and PCL. The scaffolds built showed very good definition of the pre-designed microarchitecture and were readily reproducible. While delamination occurred at larger unit cell sizes, this had no effect on the overall shape or the structural integrity of the scaffolds.

As PCL scaffolds showed better structural integrity, they were chosen for mechanical characterisation as well as in vitro studies using Saos-2, an Osteosarcoma cell line. Results showed that different scaffold designs exhibited different stress patterns and mechanical properties as well as varying rates of cell proliferation

The potential of this system lies in its ability to design and fabricate scaffolds with varying properties through the use of different unit cells and biomaterials to suit different tissue engineering applications. Experiments showed that combinations of different

polyhedral unit configurations with different biomaterials not only influenced the mechanical properties of the scaffolds, but also the proliferation rate of seeded cells.

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I INTRODUCTION

1.1 Background

For the past decade, the focus of Tissue Engineering (TE) has been on the aspect of culturing organs in the hope of replacing damaged or diseased organs in the body. In TE, expertise from the fields of biological and material sciences, and engineering are combined to develop viable biological substitutes of a tissue which helps to restore, maintain and/or improve the functions of that tissue. For matrix-producing connective tissues, the cells are anchorage-dependent and the presence of 3-D scaffolds with interconnected pore networks are crucial to aid in the proliferation and reorganisation of the cells [1, 2]. The scaffolds, which can be fabricated from natural or synthetic biomaterials [3-5], facilitate the creation of functional and structurally appropriate biological replicas of healthy versions of the required tissues. The ultimate aim is for these fabricated structures to be the basis of tissue regeneration such that patients can readily obtain implants which will not put them at risk for infection nor require them to be on life-long medication for organ rejection.

At present, scaffold fabrication is mostly done using a variety of conventional methods such as salt and particulate leaching [1, 4-6], which have been successfully applied to establish the viability of various TE applications; engineered skin has been used clinically [7] and organs successfully tested in preclinical studies include blood vessels [8] and the bladder [9]. However, these techniques rely heavily on users' skills and the applied procedure. Due to the difference in skills and procedures between individual users, the fabricated scaffolds are subject to variations and are not easily reproducible. Also, such

techniques can only produce scaffolds with a range of pore sizes. As a result, these scaffolds render the researcher incapable of making consistent analysis.

Since its inception, Rapid Prototyping (RP) technology has created a significant impact on the medical community. By combining medical imaging, Computer Aided Design (CAD) and RP, it is possible to create accurate patient-specific anatomical models [10-13] and customised prototypes of devices for a wide variety of medical applications [14-19]. This has prompted researchers to experiment with RP techniques to fabricate scaffolds which can give controlled microarchitecture and higher consistency than those fabricated using conventional techniques. However, the internal microarchitecture of scaffolds built using the original building styles and patterns supplied by RP system manufacturer are limited [20]. As such, further improvements are needed to augment the range of possible pore sizes, their accuracy and the consistency in their distribution and density. Since RP processes begin with the creation of a 3-D CAD model of the scaffold structure, one possible solution to achieve such improvements is to design the scaffold's internal microarchitecture during the CAD modelling stages before committing the scaffold designs to RP fabrication [20-27].

To alleviate the difficulty encountered in creating scaffolds with designed internal architectures, Hollister *et al* [21] introduced an image-based approach for designing and manufacturing TE scaffolds. In their work, a general program for building up a scaffold's internal architecture by repeating a unit structure of cylinders, spheres, and other entities was developed. Hollister *et al* also developed CAD techniques [22-26] for creating sacrificial moulds with designed internal channels or cavities which resembled the negative image of the final required scaffold. The moulds were used to cast

hydroxyapatite scaffolds for bone TE applications from a highly loaded “reactive ceramic suspension” following the lost mould shape forming process.

This project takes a different approach and aims to make significant improvement by employing CAD data manipulation techniques to develop a novel algorithm. This algorithm, which can be used to design and assemble a wide range of scaffold internal architectures from a selection of open celled polyhedron shapes, is to be used in conjunction with an integrated manufacturing approach [10] that combines medical imaging and RP technologies to achieve rapid automated production of pre-designed 3-D tissue scaffolds that are not only consistent and reproducible, but also patient-specific. The system is named CASTS or Computer Aided System for Tissue Scaffolds.

In contrast to the fabrication approach adopted by Hollister *et al*, which is based on the lost mould casting process, CASTS is aimed at direct fabrication of scaffolds on a RP system such as Selective Laser Sintering (SLS). By adopting a direct fabrication approach, many disadvantages associated with casting can be eliminated. Examples of such disadvantages include additional production stages (lead time) incurred in developing a suitable suspension of the required material and a mould, increased costs, increased material wastage, and risk of material contamination.

1.2 Objectives

This project aims to accomplish the following:

1. Development of CASTS for use in conjunction with an existing CAD software for automatic assembly and generation of scaffolds by:
 - Investigating and selecting the suitable geometric shapes for use as basic cellular units for scaffolds with polyhedral shapes as a starting point,
 - Building a parametric library of such basic units using the CAD software,
 - Creating an automatic algorithm to complement the library,
 - Integrating the library and algorithm with imaging software to generate patient specific scaffolds,
2. Validation of CASTS using SLS as the means of fabrication of generated scaffolds:
 - Using an industrial material, Duraform™ Polyamide, and
 - Using biomaterials
3. Verification of the use of the scaffolds as cell templates by carrying out *in vitro* experiments,
4. Characterisation of the scaffolds in terms of strength and specific cell requirements.

1.3 Scope

The scope of this project includes:

- i. Determining the effect of three SLS parameters, namely, laser power, scan speed, and part-bed temperature with relation to the quality of the scaffold.
- ii. Fabricating the scaffolds using one biomaterial which is suitable for bone cells
- iii. Carrying out *in vitro* experiments and characterisation of the scaffold patterns generated

The validation work will be carried out in two stages. Firstly, a commercially proven material, Duraform™ Polyamide, will be first used for optimisation of scaffold fabrication. Then, a biomaterial, such as Poly-ether-ether-ketone (PEEK) or Polycaprolactone (PCL), will be employed.

1.4 Organisation of The Thesis

The thesis is organised as follows:

Chapter 1 gives an introduction of the field of TE scaffolds and the challenges faced by researchers in fabrication of these scaffolds. A solution to the limitations is proposed.

Chapter 2 describes the cellular matrix of connective tissues and cell biology, discusses the current work in terms of viable TE scaffold fabrication with conventional techniques as well as RP techniques. Details of the working principle of the SLS are also included.

Chapter 3 is an overall view of the Computer-Aided System for Tissue Scaffolds (CASTS). A summary of the different modules is presented.

Chapter 4 describes in detail, the process development of the main component of CASTS, the Designer's Toolbox, from the selection of polyhedral shapes for the Parametric Library and modelling the chosen unit matrices, to the construction of the Automated Algorithm.

Chapter 5 is a case study of the femur implant created by CASTS to establish the capability of fabricating patient specific scaffolds with designed internal architecture.

Chapter 6 details the various biomaterials successfully employed with CASTS and ventures into making biomaterial scaffolds.

Chapter 7 presents the characterisation of the scaffolds through techniques such as microcomputed tomography, compression testing and finite element analysis.

Chapter 8 details the *in vitro* work done on the fabricated polycaprolactone scaffolds.

Finally, the conclusion of the project as well as recommendations for future work is presented in *Chapter 9*.

II LITERATURE REVIEW

2.1 Cell Biology

Many tissues in the body are anchorage dependent i.e. they need structural supports in order to grow and multiply. When cells are seeded onto a support structure under the right conditions, they adhere to the surface of the structure, and consequently proliferate, differentiate and grow into functional tissue [28].

Cell adhesion, while still a topic of research, is understood to be a complex process involving many internal and external forces. The process involves the initial approach of the cell and the attachment of the cell onto the substrate as well as the subsequent spreading of the cell. Some of the physical forces acting on a cell and the substratum include electrostatic forces, van der Waals forces, and steric stabilisation forces. Electrostatic forces arise due to the presence of charges on the cell and the substratum. Most intact cells have a negative charge and hence positively charged substrata would encourage cell adhesion. The charge of the substrata can also be altered by the presence of sera. However, through experiments, the influence of the electrostatic force has been found to be small in comparison to other physical forces. Van der Waals forces are the result of temporary charges due to movement of electron clouds within atoms which causes the charge to be asymmetric. This force, while relatively small within each atom, is considerable when multiplied by the large number of atoms involved. Most cell membranes are also coated with polymer chains. During the cell's approach to the substrata, pressure is built up as these polymer chains are compressed between the cell membrane and the substrate surface. Also, as water is squeezed out from the hydrated chains, osmotic pressure develops, making approach difficult [28].

Adhesion mechanisms also involve specific cell adhesion where a cell takes cues from its surroundings and bind or land on only specific types of molecules on the substrate surface. This is controlled by cell receptors, protein molecules, and proteoglycans, macromolecules believed to attach the cells and proteins on the surface of the substrate. It has been found that non-specific factors such as physical forces are only important during the initial approach and attachment of the cell to the substrate. The spreading, essential for the cell's survival and proliferation, depends on the specific cell adhesion [28].

Extracellular matrix (ECM) is a complex macromolecular assembly of molecules that are produced, processed, and assembled by resident cells. The composition of ECM is usually tissue-specific but many components of the ECM are common to most tissues [29]. In bone, the ECM constitutes of mainly collagenous proteins and other molecules which are important in structural properties as well as cell adhesion and differentiation [29-31]. Thus, the ECM not only helps to maintain tissue structure but also transmits information to attached cells [29, 32].

In vivo, the ECM serves as a reservoir for growth factors and other molecules that protect against degradation, presents these factors more efficiently to their receptors or affects their synthesis. A natural substrate can be extracted from a tissue by dissolving the other components of the tissue to extract the ECM, which can then be used for in vitro studies [33].

2.2 Bone Tissue Engineering

The bone is a complex tissue that has many essential functions in the body. It protects vital organs, provides structural support, and enables locomotion, and generates red and white blood cells [34]. Bone has mainly three types of cells. Osteoblasts, formed by osteoprogenitor cells, deposit minerals into the matrix; osteoclasts, which resorb bone; and osteocytes, which are differentiated osteoblasts. During the initial stage of bone formation, the matrix secreted by the osteoblasts undergoes calcification. As the osteoblasts become compartmentalised by the calcifying matrix, they gradually lose the ability to produce the matrix [32]. Bone remodelling (anatomy, density, and function) is governed by mechanical loading and as such, stress-shielding results in weak bone density and eventually, bone atrophy [35].

At a different level, the bone can be viewed as a composite material that has complex mechanical properties. A highly vascularised tissue, the bone is composed of mainly two types of tissues, cortical and cancellous bone. Both are a combination of mineral and biological materials but with different composition ratios. The chemical composition of the bone consists of compounds such as calcium phosphate, calcium carbonate, calcium fluoride, magnesium phosphate, and sodium chloride. These compounds contribute to the hardness and rigidity of the bone [34]. The cortical bone is strongest in compression, tension, and shear but the cancellous bone is less stiff and not as strong. Table 2.1 shows the properties of human bone [36].

Table 2.1: Properties of human bone [36]

Type of bone	Type of force	Strength (MPa)	Modulus (GPa)
Cancellous	Tension / Compression	6.6-36.2	0.13-1.08
Cortical	Tension	66-162	10.9-29.2
	Compression	167-213	14.7-24.5

Bone tissue engineering deals with researching methodologies that will result in complete regeneration of bone and restoration of its function. Current strategies include the transplantation of highly porous scaffolds seeded with cells [37]. These cells are cultured in vitro prior to transplantation to allow for proliferation, differentiation, and generation of ECM. These cultures result in formation of 3-D bone nodules. This implies that a 3-D network is a natural environment for the formation of ECM [32].

2.3 Requirements of Tissue Engineering (TE) Scaffolds

Scaffolds are necessary for growing bone tissue as they act as temporary substrates for the anchorage-dependent osteoblasts. Bone scaffolds should ideally assume the shape of the defect, provide mechanical support to the defect while healing occurs, and allow cell proliferation and tissue ingrowth into the scaffold. Seeded cells adhere to the scaffold in all three dimensions, proliferate and produce their own ECM which takes over the function of the biomaterial scaffold [38, 39].

Functional properties of the scaffold depend on the characteristics of the scaffold material, the processing techniques, and the scaffold design, which in turn decides how the cells interact with the scaffold. The scaffold must be designed to cater to the conflicting needs of tissues in terms of mechanical strength, porosity, uniformity in pore size, and complexity in three dimensions [40]. Listed below are the desired characteristics of a scaffold [6, 26, 41, 42]:

1. 3-dimensional, highly porous with an interconnected pore network for cell growth and flow transport of nutrients and metabolic waste,
2. Biocompatibility and biodegradability,
3. Suitable surface chemistry and topography for cell attachment, proliferation, differentiation, and also to encourage formation of ECM,
4. Pre-defined microarchitecture,
5. Mechanical properties to match those of the tissues at the site of implantation; for load-bearing tissue, the scaffold must provide additional mechanical support during regeneration of tissue,
6. Sterilisability.

Pore size, porosity and pore network

Pore size and porosity are the primary deciding factors for in growth of tissue in the scaffold. Increased porosity and increased pore size induce tissue ingrowth [43, 44]. Such integration of cells at the bone-implant interface increases the mechanical stability of the implant [45]. Materials often lack continuous interconnected pore networks and often, pore structure is the main limitation which prevents cell ingrowth as well as transport of molecules [42]. Macropores must be at least 100-300 microns in diameter and interconnected by micropores of minimum 10-12 microns [46].

For bone cells, the range of size for macropores is 200 to 600 μm [47]. Such macropores are necessary to facilitate cell migration, tissue growth and ECM production [38]. Studies have shown that pores of 200 μm in size caused occlusion when seeded with bone cells and nominal pore sizes of 500 μm permitted 3-D tissue growth in vitro [45].

Open structures often promote vascularisation of the tissues [42]; vascularisation has been observed to occur at micropore sizes of 30 microns and above [46]. Also, compared to isolated pores, interconnected pores improve the efficiency of nutrient supply and waste removal thereby increasing the viability of cells in the inner regions of the scaffold structure [38, 48]. Scaffolds that do not exhibit interconnected pores fail to induce bone formation in the bulk scaffold [48].

When the scaffolds have a suitable range of pore sizes, an increase in porosity is seen to cause an increase in rate of proliferation. Porosity is also found to have a stronger influence on the mechanical properties of the scaffold as compared to pore size [49].

Biocompatibility

When the scaffold construct is biocompatible with the host bone tissue, no immunological or clinically detectable foreign body reactions such as inflammations should occur [6, 38]. Temporary disturbances may appear locally if the surrounding tissue is slow in eliminating the by-products of the degradable scaffold [6, 45]. Chemicals involved in processing techniques can affect the biocompatibility of the material. Residual chemicals such as emulsifiers can leak out of the scaffold after implantation. These chemicals may be cytotoxic and harm the newly formed tissue or the surrounding tissue, hence processing techniques are important in the fabrication of scaffolds [38].

In general, it is found that composite materials improve biocompatibility and hard tissue integration. Examples include the composites of hydroxyapatite (HA) and tricalcium phosphate (TCP) [6, 45].

Surface chemistry and topography

The attachment, viability, proliferation, and differentiation of the cells are influenced by the interaction of the cells with the solid microenvironment. The degree of remodelling is dependent on host anatomy and physiology [6, 45]. When seeded onto a biomaterial scaffold, the behaviour of the cells depends on the surface topography and chemistry [32, 45, 50-52] as well as the degradation characteristics of the material [32, 50, 51].

Initial attachment of the cells onto the scaffold is dominated by the ability of the substrate to absorb proteins either from body fluids (in vivo) or the serum contained in culture media (in vitro) [52]. Cells have been found to react to different topology of surfaces and attach at specific preferred sites [53]. Theoretically, when cells are seeded onto the

substrate in vitro, they will proliferate and synthesise an osteoinductive matrix onto the surface of the scaffold thereby facilitating the growth and differentiation of tissue even if the initial batch of cells does not survive the implantation of the scaffold [54]. How the cells then attach to the scaffold will dictate their subsequent behaviour; strong adhesion of cells to substrate is usually associated with proliferation [38]. For bone tissue, strong adhesion of cells onto scaffold is desired as insufficient adhesion of tissue onto polymer causes gap formation during load bearing [46]. This can result in malformed tissue which might not be able to perform the original functions of the organ.

Mechanical properties

One crucial requirement of load bearing implants is that the elastic modulus of the implant must match that of the native tissue of bone [32, 38, 46, 55]. Tissue ingrowth is positively affected by a comparable modulus [46]. This means that while the implant must be strong enough to withstand physiological stresses, it should allow load sharing with the growing bone [38, 44]. If the implant has a much higher value of modulus, stress shielding occurs [56]. As bone is a dynamic tissue, the lack of mechanical stimulus causes the bone cells to be resorbed hence, resulting in biological damage to the bone [38, 45, 56]. One strategy is to design a scaffold which should only be resorbed only after the tissue is fully remodelled by the host tissue and is structurally stable [38]. On a smaller scale, the stiffness of the material may influence the cytoskeleton of the cells to induce cell spreading and division [28, 38].

Pre-defined microarchitecture

A highly porous scaffold does not always encourage cell proliferation, differentiation, and reorganisation [57]. Scaffolds with uncontrolled microarchitecture produce vast amounts of regenerated tissues which, however, possess poor biomechanical properties [58]. Uneven pore sizes can result in non-uniform cell density within the scaffold. Occlusion may also occur at smaller pore sizes and this will prevent cellular penetration and matrix formation within the scaffold [44].

Sterilisability

The scaffolds must be sterilisable to prevent infection after implantation. The method of sterilisation should not interfere with the biocompatibility of the material nor alter its inherent biochemistry [38].

2.4 Conventional Techniques of TE Scaffold Fabrication

There are two ways of incorporating cells into scaffolds [59]:

1. Seeding cells onto the scaffold after scaffold fabrication (Preformed scaffolds)
2. Incorporating of cells into the scaffold fabrication process (Cell incorporated scaffolds)

2.4.1 Preformed Scaffolds

Preformed scaffolds are most widely investigated for cell transplantation. To make them more biocompatible, any residual toxic substances left over from processing are removed by leaching the scaffold in water for several days [38]. As discussed in the previous section, surface features and architecture are very important in tissue engineering scaffolds. Earlier research has also shown that cells thrive better on foamed structures than on flat surfaces [60]. Hence, scaffold fabrication techniques are aimed at producing porous structures. There are several conventional methods of producing preformed scaffolds. Commercially available scaffolds such as DegraPol[®] scaffolds [61] and BD[™] Three Dimensional scaffolds [62] are fabricated using such methods.

Fibre bonding

One of the pioneer techniques of scaffold fabrication, this method uses PGA fibres to form 3-D structures [63]. Two different variations of this method have been developed and both are found to produce highly porous fibre foams with interconnecting pore networks which are suitable as scaffolds. The main disadvantage of this method is in the removal of the toxic solvents used during the process. As this step takes a long time, the scaffolds cannot be used for immediate clinical applications [64].

Solvent casting/ Particulate leaching

In this method, water-soluble porogens such as common salt, are placed in a petri dish. The polymer is dissolved using an organic solvent and cast into the petri dish. Upon evaporation of the solvent, the scaffold is leached to get rid of the salt crystals. The porosity is thus dependent on the quantity of salt and the pore sizes are reflective of the size of the salt crystals. High porosity and interconnected networks are obtained with 70 weight percent of salt [65]. In one study, salt crystals are fused prior to casting in an attempt to improve interconnectivity between the pores of a poly(lactide-co-glycolide) or PGA scaffolds. While this aim is achieved in the PLG scaffolds, the pore structures are found to be disorganised [66]. In another variation, compression moulding is used instead of the petri dish in a bid to improve the method. This produced scaffolds with uniform surface morphology and allowed for better control of scaffold thickness. However, there are concerns raised regarding the high temperatures involved during compression moulding which might cause the polymer to degrade. Another concern is the use of solvents which might cause cytotoxicity if not properly removed [64].

Gas foaming

This method is developed to eliminate the need for organic solvents. Solid discs are first fabricated via compression moulding. These are then placed in a chamber and exposed to high pressure CO₂ for 72 hours during which the pressure is rapidly decreased to the atmospheric pressure. Scaffolds thus produced have porosities of up to 93% and pore sizes of up to 100µm [67]. The main disadvantage lies in the absence of interconnected pores especially on the surface of the foam. Another approach combines this and particulate leaching methods to produce scaffolds. Ammonium carbonate added to the dissolved polymer results in a putty-like mixture which is easily shaped and moulded.

Porosities of up to 90% and pore size of range 200-500 μ m can be obtained using this method. The same concerns are raised as to the use of organic solvents and the long-term effects of the residue of ammonium carbonate on cells [64].

Phase separation/ emulsification

This method makes use of the immiscible properties of liquids to form pores. In the first approach, the polymer, dissolved using an organic solvent, is mixed with water and cast into a mould. The mould is quenched with liquid nitrogen and freeze dried, resulting in the removal of dispersed water and solvents. Scaffolds obtained have high porosities (up to 95%) but pore sizes are less than 50 μ m [64]. Continual adjustments made to this method have increased the number of interconnected pores within the network as well as the pore sizes. However, like other methods, the use of organic solvents remains a limitation [60, 64].

2.4.2 Cell Incorporated Scaffolds

Injectable scaffolds

Bioresorbable injectable polymers are comparatively easier to fabricate into scaffolds as they can be formed into all types of shapes and sizes. In this method, cells are mixed with the polymer itself and injected into the defective area (see Figure 2.1). The material must be polymerised or cross-linked to obtain sufficient mechanical strength for load-bearing implants. This is achieved through addition of reagents such as initiators. Porogens are also added to the scaffold such that after implantation, the body fluid environment causes the porogens to leach out, leaving a porous scaffold [38].

Figure 2.1: Production of injectable scaffolds

This set of procedures results in additional constraints. It is important that all reagents must not be toxic and porogens must not harm local tissue during the *in vivo* leaching process. Reaction parameters such as temperature and pH also have to be carefully controlled to prevent adverse effects on embedded cells [38].

2.5 Application of RP in TE Scaffold Fabrication

Conventional techniques of scaffold fabrication rely heavily on user skills and experience such that there is poor repeatability in between users. Processing parameters are often inconsistent and inflexible thus resulting in highly inconsistent micro and macro structural properties in the scaffold. Use of organic solvents may have harmful effects on the cells and cause the cells to die or mutate. Porogen particles employed to induce pores may not be completely removed, making the process inefficient in terms of porosity. Most conventional processes are also limited to producing scaffolds with simple geometry which may not satisfy the geometric requirements of the defect [44].

Although RP techniques can potentially address most of the macro and micro structural requirements of TE scaffolds, only a few of the RP systems have been used for scaffold fabrication to date [44]. Leong *et. al.* recognized that most of the work done in Tissue Engineering scaffolds using RP systems have been in modification of the RP systems to suit the specialised application of biomedical engineering [20]. The following section discusses the application of RP in TE scaffold fabrication.

3-Dimensional Printing (3DP)

3DP is the most widely investigated system with regards to its possible application in TE. 3DP makes use of solutions to bind powder together to form parts [68]. Poly (L-lactic acid) (PLLA) and poly glycolic acid (PLGA) scaffolds fabricated using 3DP which exhibited pore sizes of 40-150 μ m and porosities of 75-90% were successfully tested in vitro. In the case of starch-based scaffolds, it is necessary to infiltrate them with copolymer solution to increase strength and water resistance. Highly porous structures with a high degree of pore interconnectivity can be achieved by varying printing speed,

flow rate, drop position of liquid binder, particle size distribution, and composition of the powders [20]. 3DP has also been used to fabricate PLGA scaffolds through the use of moulds and particulate leaching using sucrose as porogen. While this method was proven to produce viable scaffolds, the lengthy process makes it tedious and ineffectual [69].

The main advantage of the 3DP system is the possible range of biomaterials. Also, as the process is carried out at room temperature, thermally sensitive materials can be used for fabrication. The disadvantages are in the standard building styles, limited pore sizes, and the widely distributed range of pore sizes [20].

Fused Deposition Modelling (FDM)

FDM is an extrusion-based system. The FDM consists of two heads, one for the model and one for the support material, that are fed through spools and heated up to melting temperature prior to extrusion [68]. FDM fabricated polycaprolactone (PCL) scaffolds had pore sizes of 160-700 μ m and 48-77% of porosity. Fibroblast cells seeded onto scaffold showed complete ingrowth after a few weeks of culture. Degradation tests proved that the process did not affect the material properties [70]. However, FDM is limited to thermoplastics with low melting points and low melt viscosities. Another difficulty is in extruding the materials (in the form of powders, pellets and flakes) into filaments with good diametrical accuracy [17]. As a result, many variations of the FDM system have been developed to overcome the limitations.

One such system is Precise Extrusion Manufacturing which can accept not only filaments but also powders, pellets and flakes [71]. Another example is TissForm, an adaptation of the FDM machine to produce TE scaffolds. The process, unlike FDM, makes use of a

non-heating liquefying processing of materials so that the properties of the biomaterials are better maintained. Composites of Poly (L-lactic acid) (PLLA) and tricalcium phosphate (TCP) have been used to fabricate TE scaffolds with the system [72].

Too *et al* [73] and Hutmacher *et el* [6, 74] made use of the lay down patterns of FDM to fabricate scaffolds with pre-determined architecture. While the scaffolds exhibited uniform pore sizes, high porosity, and comparable mechanical properties to that of the human spongy bone, the internal microarchitecture of the scaffold was only limited to repetitions of simple geometric shapes [20]. It was also found that pore morphologies along the different co-ordinate axes (x, y, and z) were not consistent [6, 57, 58]; this was due to inherent operation parameters of the machine. Another disadvantage of the FDM is the need of support for overhanging features [68].

Selective Laser Sintering (SLS)

In selective laser sintering, the powder bed is selectively scanned by a CO₂ laser to form parts [68]. PMMA-coated calcium phosphate has been successfully sintered in SLS to fabricate scaffolds. The PMMA was subsequently removed in a furnace, leaving a porous structure of ceramic. Inorganic cement was then infiltrated into the structure to increase the strength and density of the scaffold. The implant was found to integrate with bone in vivo [20]. Rimell *et el* [75] attempted to sinter ultra high molecular weight Polyethylene for clinical applications but found that the laser caused the material to degrade.

Sintering of Poly-Ether-Ether-Ketone (PEEK), PLLA, and PCL powder stocks had different degrees of success. Microporosity of the specimens was varied by changing laser power (or energy density), scan speed and part bed temperature. High porosity and

interconnectivity were obtained for all specimens [76]. Similar to 3DP, SLS is capable of fabricating complex 3-D structures, including channels and overhanging features, as the powder acts as a self-support [20, 68].

Stereolithography Apparatus (SLA)

SLA, the pioneer RP technique, is based on the use of a laser to photopolymerise liquid resins that are curable through exposure to UV light [68]. Due to the limited availability of photocurable biomaterials, SLA is largely unexplored as a means of fabricating tissue engineering scaffolds. Cooke *et al* demonstrated that it was possible to fabricate polypropylene fumarate scaffolds for critical-sized bone defects using SLA. However, there was no data on in vitro or in vivo studies [77]. In another study, a new biodegradable, photopolymerisable copolymer, acrylated capped poly- ϵ -caprolactone-co-trimethylene carbonate was developed to fabricate microarchitectural features using the SLA [78].

Inkjet printing

In inkjet printing systems such as Model Maker II (MMII), scaffolds are usually fabricated through the indirect method. These systems have dual print heads that deliver different materials, one for model and the other for support. The heated material solidifies upon impact cooling. MMII is used to produce moulds for scaffold manufacturing. The lost mould method is used to cast out the scaffolds which are made of naturally occurring materials such as tricalcium phosphate [79] and collagen [80].

Other RP processes

In recent years, researchers have attempted to develop RP systems that are aimed solely at processing biomaterial implants such as scaffolds. One example is the 3D plotter. In this process, hydrogel parts are fabricated through the dispensing and subsequent reaction of the material with another chemical. Oligoetherurethanes were successfully processed using this method [59, 81, 82]. The bioplotter is now being commercially marketed [83]. Another similar example is the desktop RP robotic dispensing system (RPBOD) developed by Geng *et al.* This system makes use of a pneumatic dispenser to deposit gel droplets. While this method is novel, the preparation of chitosan gel still involves the use of an inorganic compound, acetic acid. This poses the same concerns about the toxicity of the scaffolds [84]. Both of the above-mentioned methods are limited to fabrication of soft tissue scaffolds as only hydrogels can be used.

A more radical technique, organ printing, directly incorporates cells and cell aggregates into gels such that the resulting scaffold is in effect a complete organ [85]. As cells are extremely sensitive to temperature changes and stresses, the concern remains that they maybe functionally or otherwise damaged during the process.

Image-based techniques and improvements on scaffold design

As there are many parameters involved in scaffold fabrication, conflicts of interests can sometimes occur. One example is the linear relationship between internal surface area and porosity both of which are necessary for tissue ingrowth. Previous studies have mostly focused on measuring the mechanical properties of the scaffolds after fabrication and therefore, scaffolds produced thus far are not pre-designed for specific tissue

requirements, such as mechanical properties and pore sizes. Hollister *et al*, combining computational optimisation procedures and RP techniques, carried out a study which can produce scaffolds with design constraints while maintaining mechanical requirements. This study, however, was confined to matching mechanical properties with the overall scaffold architecture [26].

Hollister *et al* [21] came up with another novel way of producing scaffolds by combining conventional techniques (casting) with RP techniques and imaging applications. The external scaffold shape and pore architecture were designed using an image-based method. Up till now, researchers have only quantified scaffolds as micro pores ($<100\mu\text{m}$) and macropores ($>100\mu\text{m}$). Taboas *et al* [86] further defined pores as global pores, large pores which are incorporated into the design (Figure 2.2), and interconnected local pores, smaller pores which are created as a result of processing techniques such as porogen leaching. The sizes of local pores are not entirely predictable but are defined to be smaller than $300\mu\text{m}$ in diameter.

Figure 2.2: Image-based design mould for scaffold casting [86]

Using this method, it is possible to produce scaffolds with pre-defined microarchitecture and consistent global pore sizes which can also match the implant size geometry. However, the simple geometric shape of the designed internal architecture limits the scope of application. Another disadvantage is the use of organic solvents which may be harmful to the cells in the case of residues present in the scaffold.

Alternatively, to achieve regular microarchitectures within scaffolds, researchers have experimented with the use of patterns. The inherent ability of FDM [44] and 3-D plotter [82] to produce regular three dimensional grids with variable spacing has been reported. Lam *et al* designed simple geometric shapes for use with three-dimensional printing [87]. While the scaffolds thus produced exhibited uniform pore sizes and high porosity, the internal microarchitecture of the scaffold was only limited to repetitions of simple geometric shapes. Hence the challenge of developing a comprehensive database of pore shapes remains.

2.6 Scaffold Processing Using Selective Laser Sintering

There are many pre-requisites for scaffold fabrication techniques. Processing techniques should not change the chemical properties of the material nor cause any degradation in its mechanical properties. The pore sizes and morphologies should be as user-defined and readily reproducible when the same set of parameters are applied [44].

Phung [88] compared four different RP systems for use in TE scaffold fabrication, namely, Stereolithography (SLA), Model Maker II (MMII), 3-D Printing (3DP) and Selective Laser Sintering (SLS). The main advantage of SLS is in the possible range of materials that can be used with the system including biomaterials. SLA and MMII, though capable of higher dimensional accuracy, require materials to undergo a phase change which might affect the properties of the materials and render them unsuitable as implants. It is also observed that MMII and 3DP have much lower part strengths than that of SLS fabricated parts. Low part strengths are undesirable, as TE scaffolds need to have comparable strengths to that of the tissue. Even though SLS has more parameters than the other systems, many of them do not influence part quality greatly and only a few important parameters need to be varied to obtain the desired part quality. Surface roughness of SLS too is advantageous in the fabrication of the scaffolds as uneven surface topography encourages cell proliferation [44, 89].

A point to note however is that during sintering, over heating must be avoided to prevent depolymerisation which can result in production of monomers and low molecular weight oligomers. These compounds degrade faster than polymers hence leading to undesirable chemical and biological effects [90] when implanted in vivo.

Process

SLS is a powder-based RP system. As with all other RP systems, its principle is based on additive formation of parts. The main components of the system are: two side powder cartridges, a platform with variable height, heaters, and a Carbon Dioxide laser beam [68, 91, 92].

A 3-D part is first imported into the STL (Stereo Lithography) format commonly used by RP systems. The SLS slices the part into thin layers. An in-built software allows the user to control the part placement and the various parameters of the build such as part-bed temperature. The part-bed temperature is usually set to either the glass transition temperature (for amorphous materials) or a temperature just below the melting point (for crystalline polymers). The two heaters raise the temperature of the part-bed to the set value such that once the laser beam traces out the layer of the part profile, the temperature of that region becomes high enough to cause the powder to become molten and form a structure [68, 92]. Other parameters involved in part fabrication include laser power, powder layer thickness and scan speed [91-93].

The SLS cycle is illustrated in Figure 2.3 with four condensed steps labelled 1 to 4.

1. The piston of the powder cartridge (the left powder cartridge in the figure) pushes up the powder so that there is sufficient powder above the part bed for one layer.
2. The roller sweeps the powder across the part bed.
3. The laser sinters the part profile of the specific layer.
4. The part bed lowers by the thickness of one layer and the other powder cartridge (right) rises up simultaneously. The cycle is repeated until the part is completely sintered.

Figure 2.3: Selective Laser Sintering™ System by DTM Co.

2.7 Biomaterials

For a biodegradable polymer to be used as implant material, its degradation should be at an adjustable hydrolysis rate with non-toxic water-soluble molecules as its final products. The polymer should be capable of undergoing surface erosion which means that hydrolysis in surface layers must be faster than that in the bulk material and hence, the polymer must be hydrophobic to prevent water from entering. The polymer must also contain linkages that can go through rapid hydrolysis such as anhydride (—O—) bonds and esters (—O—C=O—) [94].

One major challenge in this area is the synthesis of scaffolds that, when in the presence of appropriate media and reactor conditions, will secrete extracellular matrix and form tissue [32]. Naturally occurring materials such as collagen or chitosan are commonly used in tissue engineering applications. However, such materials cannot be processed using laser sintering techniques [33]. On the other hand, the materials employed in the SLS are commercially available polymers such as Duraform™ Polyamide (Nylon 12). Such materials however, are not biocompatible and cannot be used in the fabrication of tissue engineering scaffolds. As a result, there is a need to find alternative materials that, while biocompatible, can be fabricated with the SLS system i.e. sintering at high temperatures should not alter the characteristics of the material so that the biocompatibility of the material is maintained.

Biocompatible synthetic polymers offer a number of advantages over natural polymers for use in tissue engineering scaffolds. Not only can the mechanical properties be tailored to suit the requirements, the polymers can also be shaped and morphological details added

in to induce cell ingrowth [64, 95, 96]. The properties and structures of such polymers are also reproducible, making them suitable for use as scaffolds [33, 97].

The ideal polymer for this application should have the following characteristics [94, 95]:

- ❑ It does not change the morphology of cells nor cause inflammation.
- ❑ Upon degradation, it only secretes non-toxic water-soluble molecules that are easily metabolised/excreted and will not harm the tissue.
- ❑ It has mechanical properties that can be varied by modifying the structure.
- ❑ It has an acceptable shelf life.
- ❑ It is easily processed into the final product form.
- ❑ It is easily sterilised.
- ❑ It can be synthesised into powder form.
- ❑ It has an adjustable hydrolysis rate.
- ❑ It is capable of undergoing surface erosion.

Other considerations include physical properties such as the glass transition temperature, T_g . Most polymers are semi-crystalline as it is difficult for a polymer to be fully organised into a crystalline structure. When implanted, the polymer is exposed to body temperature. A polymer with a value of T_g around the body temperature is then likely to become more ductile than at room temperature. This, in turn, can influence a change in the mechanical and degradation properties of the implant [95].

Tan *et al* investigated 5 biomaterials, 4 polymers and a ceramic, for use in scaffolds fabricated by SLS, namely, Poly- ϵ -caprolactone (PCL), Poly (vinyl alcohol) (PVA), Poly-ether-ether-ketone (PEEK), Poly (L-lactic acid) (PLLA), and Hydroxyapatite (HA). The 3 main parameters of the SLS system, fill laser power, fill scan speed and part bed

temperature, were varied to obtain viable parts using these materials. While it was not possible to sinter HA because of the high temperatures required, the biocomposite of PEEK blended with HA was successfully sintered [98].

In this section, various biomaterials are examined with regards to their suitability for use with the SLS as well as their application as tissue engineering scaffolds.

2.7.1 Poly(L-lactic acid) (PLLA)

Poly(L-lactic acid) or PLLA belongs to the family of polyorthoesters. PLLA has low elongation and exhibits high tensile strength and consequently, a high modulus that makes it suitable for load bearing applications [95]. Mechanical properties of this material can be changed by varying the ratio of the flexible and rigid diols used in the synthesis. Most attractive is its initial degradation products which are neutral [94].

Although PLLA contains hydrolytically labile linkages, it is also highly hydrophobic and therefore very stable and can be stored at room conditions without moisture seeping in. Initially developed as suture material, PLLA is now commercially used in bioerodible devices in drug delivery and interference screws used for fracture fixation [94, 95]. It has also been used to fabricate bone TE scaffolds via a variety of conventional methods as well as FDM [72].

Although a few studies have shown that acidic degradation products of PLA polymers, one of which is PLLA, maybe the cause of local or systemic reactions in the body, PLLA has been widely accepted to be biocompatible [95, 96, 99]. PLLA also has a slow rate of

degradation such that more than 2 years is required to be completely reabsorbed [95]. This is attributed to its high glass transitional temperature (56°C) [100]. The biodegradation mechanism starts with random hydrolysis which results in a decrease in molecular weight, followed by a reduction in mechanical properties and mass loss. Being bioabsorbable, the final degradation products are assimilated by natural pathways (metabolism, excretion) [99] and no significant amounts of accumulation of degradation products of PLLA have been reported [95, 96]. One disadvantage of PLLA is in sterilisation; use of γ -radiation is not possible as PLLA is especially sensitive to such radiation while autoclaving will degrade the material [95].

2.7.2 Poly- ϵ -caprolactone (PCL)

PCL is a semi-crystalline polymer which is most widely used in the family of lactones. It has a high solubility and is also compatible with a range of other polymers. Therefore, it has been used in blends for biomedical applications. As the rate of degradation is very slow (two to three years), it is useful for long-term applications such as drug delivery systems. It is considered to be non-toxic and compatible with body tissues [96].

PCL has a melting point of approximately 60°C and has a glass transition temperature of -60°C. Hence, the polymer is at a rubbery state at room temperature [101]. Its crystallinity decreases with the increase in molecular weight [102]. PCL has high thermal stability and only decomposes at temperatures of above 350°C [103]. PCL is being used as degradable sutures in clinical applications. To overcome the slow rate of degradation, PCL is often synthesised into copolymers [104]. Degradation of PCL occurs in two modes: degradation by micro organisms [105] and by hydrolysis under physiological conditions [106]. Hydrophobic nature of PCL translates to slower degradation rate than other members of

the aliphatic polyester family such as polylactide (PLA) polyglycolide (PGA) [106]. PCL is not commonly used in orthopaedic applications due to its low strength [107].

Although PCL is usually synthesized in pellet form, it is also available in powder form. Not only has PCL scaffolds been fabricated through conventional methods (particulate leaching) [108], PCL has also been researched for use in tissue engineering scaffolds using rapid prototyping, however, the techniques employed have been centred on processing of PCL in pellet form [103, 109], hence there is little data on PCL scaffolds developed from powders. Methods of producing biocomposites of PCL/HA include using an extruder to blend the polymer (PCL) with the reinforcement material (HA) [110] and grafting of PCL to HA by ring-opening polymerisation of PCL in the presence of HA particles [111].

To date, Ethylene oxide (EtO) gas [101] or ethanol is used to sterilise PCL. While this is a common practice, the use of such organic solvents maybe harmful to cells if not properly leached out.

2.7.3 Poly (vinyl alcohol) (PVA)

PVA is a bioinert material. Being a semi-crystalline polymer, the degree of cross-linking influences its physical properties such as glass transition temperature and melting point. These values also vary with the presence of water. PVA is also water-soluble but its solubility depends on the degree of hydrolysis and the degree of polymerisation. In general, the solubility of PVA decreases with an increasing degree of hydrolysis and a decreasing temperature [112-116].

Hydroxyl groups present in PVA can be modified to attach chemical molecules which aid in adhesion and growth of cells. However, as PVA is commonly synthesised in hydrogel form, there is difficulty in getting cells to be attached to PVA scaffolds. This is because hydrophobic scaffolds allow for better cell attachment and matrix deposition and the high hydrophilicity of hydrogel hampers deposition of extracellular matrix [117]. The scaffolds can be modified with cell adhesion proteins to better facilitate the attachment of cells onto scaffolds [33, 117]. Attaching proteins onto substrate surfaces is done by covalent linking of protein molecules with PVA [117]. PVA has been used in making TE scaffolds for heart valves and soft-tissue applications [113, 114, 117, 118]. Production of PVA powders by spray drying is time-consuming and ineffective as the yield is very low.

2.7.4 Poly-Ether-Ether-Ketone (PEEK)

PEEK is a bioinert material [119]. It is not biodegradable as it is hydrophobic and hence, possesses poor wettability. The lack of cytotoxicity and mutagenicity of PEEK has been proven in previous research [51]. Its advantages lie in its strength and stiffness, which are compatible to that of the bone [56, 119] as well as its stability during sterilisation using autoclave [56]. Conventionally processed through shaping or forming operations, the mechanical properties of PEEK and its composites are dependent on the thermal processing conditions [119].

Barton *et al* [120] found that though PEEK is a non-biodegradable polymer, it had the same rate of proliferation with biodegradable polymers such as poly(orthoester) (POA) and poly(L-lactic acid) (PLLA) when used as a substrate for bacterial cells. In vitro

cytotoxicity tests also showed that PEEK did not cause any marked effect in morphology of rat osteoblast cells when incubated for 48 hours. The same set of experiments indicated that PEEK stimulated osteoblast protein content and thus may encourage ingrowth of tissue [56].

In SLS, high temperatures are reached when polymers are sintered to form parts. As a result, the polymer used must be one that can retain its properties even at high temperatures. PEEK (Poly-Ether-Ether-Ketone) degrades at temperatures between 550-600° C in a nitrogen atmosphere [121]. While the thermal stability of the material is found to be significantly reduced in the oxidised atmosphere, this need not be a consideration as the polymer is only exposed to momentarily high temperatures in the inert chamber filled with nitrogen gas.

2.7.5 Hydroxyapatite (HA)

To date, the most successful bone grafting material is autogenous cancellous bone. There are shortcomings however in the morbidity involved as well as the risk of infection and blood loss [122]. Hence, there is a need for an alternative material which displays the same properties such as osteoinductivity and osteoconductivity. This led the researchers to explore the possible use of a bioactive ceramic, hydroxyapatite (HA), a phase of the bone that occurs naturally [42, 123, 124]. Calcium phosphate-based materials such as HA have been employed in the medical and dentistry applications for many years [39, 90]. Studies have shown that HA is biocompatible with hard tissues of human beings such as bone and also displays osteoconductive properties [42, 45, 123, 124].

Levy *et al* fabricated a custom-made HA bioimplant of the orbital floor based on CT images and concluded that the rate of degradation of the implant may be increased by means of a highly porous internal architecture [15]. Ono *et al* successfully used large HA implants to treat complex cranial bone defects. The same study, however, pointed out that the pure HA implants have low strengths and being brittle, are prone to crack upon impact [16]. Therefore, pure HA ceramics are not mechanically suitable as bone tissue implants, which are required to bear loads.

As it is known that incorporation of another material can change the properties of a given material [42], research is thus carried out in an effort to combine HA with other types of materials which are either commonly used for implants, for example, titanium [90], or biocompatible polymers [123, 125]. Investigations have been carried out to ascertain the properties of hydroxyapatite-based materials, polymer matrices and combinations of both types of materials [122].

When combined with polymers, HA, a bioceramic, becomes easier to shape and also less brittle [123]. Studies also showed that the addition of HA improved the hydrophilicity of biodegradable polymers and enhanced the adhesion and proliferation of osteoblast cells onto the substrates [50, 125]. As a result, biocompatible polymer/ bioceramic composites are promising materials for use as bone implants.

2.7.6 PEEK-HA Composites

In vitro experiments revealed that incorporation of bioceramic particles in biopolymers rendered the composites bioactive and there was a significant increase in the ability of the composites to induce the formation of bone-like apatite on the surface [119, 126, 127]. A material is said to be bioactive when the tissue forms an interfacial bond with the implant [128]. It must be noted that the HA particles need to be exposed to elicit the bioactivity of the composite [119]. Such composites have also been found to support the proliferation, differentiation, and mineralisation of human osteoblast-like cells [127]. However, these composites were fabricated with conventional methods and thus, the materials used may not be applicable with RP techniques.

SLS composite materials are already available commercially. Glass or metal particles (which have high glass transition temperatures or melting points) are physically blended with a polymer such as polyamide. The polymer, melted by the laser, acts as a binder and entraps the other material within its sintered network. By this principle, it is possible to blend PEEK and HA powders together and sinter the composite such that the result is a porous interconnected network of PEEK with embedded HA particles. Tan *et al* [98] blended PEEK with varying weight percentage of HA ranging from 10-40% and successfully sintered the composite. They noted that once the weight percentage of HA exceeded 40%, the sintered part became fragile and its structural integrity of the part was compromised.

An investigation on the properties of conventionally fabricated PEEK-HA composites (via injection moulding) showed that an increase in the amount of HA in the composite

led to a decrease in ductility of the composite, as HA is a brittle material. The tensile properties of the composite were found to be dependent on the content of HA. The Young Modulus of the composite lied within the low to mid range of the natural bone modulus (3-30 Gpa) depending on the amount of HA present [119].

2.8 Computer-Aided Design: Pro/ENGINEER

Pro/ENGINEER is a popular graphic software that is used to design products for manufacturing. Data exchange with other softwares and manufacturing systems can be done with ease, as it is compatible with many industrial standard data formats. Its popularity is owed to the intuitive interface and its flexibility in easy review and modification of generated models. Apart from allowing users to draw digital parts, Pro/ENGINEER software has peripheral modules that allow the creation of interactive interfaces so as to allow users with little or no experience in graphic design to generate different dimensioned parts of the same generic design.

Furthermore, Pro/PROGRAM, one of the modules, enables the user to create relationships between different parameters of the parts called relations. Each relation is a combination of parameters, operators, functions, and conditional statements. Table 2.2 is a summary of terms used in the program. By combining the relations and programming language, the user is able to control the dimensions of parts as well as automate the regeneration of the assembled parts.

Table 2.2: A summary of terms used in the Pro/PROGRAM

	Type	Example
Data types	Integer	0,1,-1
	Real	-9.0,0.5,4.25
	String	A-Z, 0-9, +, \$
	Boolean	True, false
Parameters	Symbolic	D1, d10
	User-defined	cell_length, strut_radius
Operators	Arithmetic	Addition (+), Division (/), Parenthesis (())
	Assignment	cell_length=1
	Comparison	Equal to (==), greater than (>)
	Logical	AND (&), NOT (!, ~)
Functions	Mathematical	Sine (sin ()), Cosine (cos ())
Conditional statements	if {} else {}	if <condition> <--relations - -> else <condition> <--relations - -> endif

When a user creates a part, a design listing, similar to that shown in Figure 2.4, is generated. Each feature in the part has a set of parameters which are represented by symbolic dimensions. From Figure 2.4, by adding relations between lines 8 and 9 as well as changing the symbolic dimensions of the part (lines 48-50), the design listing can be modified to include user-defined parameters and edited to work like a program such that the modelled structure reflects the changes in design specifications.


```

1  VERSION I-02-18
2  REVNUM 43
3  LISTING FOR PART CUBE
4
5  INPUT
6  END INPUT
7
8  RELATIONS
9  END RELATIONS
10
11 ADD FEATURE 1
12 INTERNAL FEATURE ID 1
13 TYPE = DATUM PLANE
14 NAME = DTM1
15
16 END ADD
17
18 ADD FEATURE 2
19 INTERNAL FEATURE ID 3
20 TYPE = DATUM PLANE
21 NAME = DTM2
22
23 END ADD
24
25 ADD FEATURE 3
26 INTERNAL FEATURE ID 5
27 TYPE = DATUM PLANE
28 NAME = DTM3
29
30 END ADD
31
32 ADD FEATURE 4
33 INTERNAL FEATURE ID 7
34 PARENTS = 1(#1) 5(#3) 3(#2)
35
36 PROTRUSION: Extrude
37
38 NO. ELEMENT NAME  INFO                                STATUS
39 --- -----
40 1 Attributes  One Side                                Defined
41 2 Section    Sk. plane - Surface DTM2 of feat #2 (DATUM PLANE) Defined
42 3 Direction
43 4 Depth      Blind, depth = 1.5                        Defined
44
45 SECTION NAME = S2D0001
46
47 FEATURE'S DIMENSIONS:
48 d0 = 2
49 d1 = 4
50 d2 = 1.5
51 END ADD
52
53 MASSPROP
54 END MASSPROP

```

Figure 2.4: Design listing for a rectangular part

2.9 Research Methodology

Earlier in this chapter, conventional methods of scaffold fabrication have been critically reviewed. It is noted that none of these techniques have been able to fabricate scaffolds of good consistency and reliability. Even with the introduction of Rapid Prototyping techniques, scaffold design has been limited to a small range of basic pore shapes. This has made it impossible to launch a comprehensive investigation to study the relationship between pore sizes, pore shapes and cell proliferation and differentiation.

It must also be recognised that tissue engineering scaffolds need to be customised to suit individual patients and hence, the use of medical imaging is necessary in scaffold design. As a result, it is proposed that CAD (computer-aided design) strategies be combined with medical imaging and rapid prototyping techniques to create a system to custom design and fabricate scaffolds. A series of tests can be carried out on these scaffolds to establish the inter-relationship, if any, between pore shapes and sizes and their influence on cell proliferation, differentiation and three-dimensional tissue growth.

It is proposed that the Selective Laser Sintering system, SinterStation™ 2500, be first used for fabrication of scaffolds designed using this system. Being a powder-based system, the SinterStation is advantageous as it does not require any extra support and hence, is suitable for free-hanging structures. Recent research has yielded biomaterials suitable for sintering. The three biomaterials are: Poly-ether-ether-ketone (PEEK), biocomposite of poly-ether-ether-ketone and hydroxyapatite (HA), and polycaprolactone (PCL).

Although PEEK is a bioinert material, it will be useful as a test material to validate the system, as it is readily available in powder form. PEEK scaffolds can also be used as controls in *in vitro* and *in vivo* experiments. In biocomposite scaffolds, PEEK particles act as a binder to HA particles, which only sinter at very high temperatures. PCL has good potential as a scaffold material as it has been fabricated using another RP technique, Fused Deposition Modeling. The other advantage is that it is FDA approved for use in clinical applications.

Figure 2.5 illustrates the research methodology adopted for this project.

Figure 2.5: Research methodology

III COMPUTER AIDED SYSTEM FOR TISSUE SCAFFOLDS (CASTS)

3.1 Introduction

As discussed in earlier chapters, even with the incorporation of RP systems, the work carried out on TE scaffolds has been restricted due to many limitation factors, one of which is the lack of variety in patterns. What is required is a comprehensive system which can:

1. Provide the users with a database of designs to choose from,
2. Generate scaffolds of different parameters, and
3. Customise scaffolds according to patients' specifications.

The aim of this project, therefore, is to develop such a system which can satisfy these requirements. The prototype system is named the ***COMPUTER AIDED SYSTEM FOR TISSUE SCAFFOLDS*** or ***CASTS***.

3.2 CASTS Architecture

At the centre of CASTS is a graphics workstation which runs Pro/ENGINEER, a 3-dimensional CAD/CAM system. CASTS has 3 separate modules, namely, the input module, the designer's toolbox and the output module. The interaction between each module and the central workstation is demonstrated in Figure 3.1.

Computer Aided System for Tissue Scaffolds (CASTS)

Figure 3.1: Modules of CASTS

3.3 Input Module

Besides the standard hardware and software for computer systems, CASTS also requires the use of imaging software such as MIMICS¹, Materialise's Interactive Medical Image Control System. This is to convert raw patient data obtained through Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) scan to surface files of IGES (Initial Graphics Exchange Specification) format. The IGES format serves as a neutral data format to transfer the design to a dissimilar system.

3.4 Designer's Toolbox

Similar to a workman's toolbox, the designer is provided with a variety of useful tools to customise the scaffold to suit design requirements. The Pro/ENGINEER² software is selected for this work mainly to exploit its parametric-based, associative-design features and tight integration with a complete suite of specialised engineering applications such as Finite Element Analysis (FEA) and Computer Aided Manufacturing (CAM) interfaces. In addition, the software offers flexibility in program editing to cater for revisions and future expansion. Although only one CAD software package, Pro/ENGINEER, is presented in this work, it should be noted that the algorithms developed is sufficiently generic to be easily adapted for most other commercialised CAD packages.

¹ MIMICS™ version 6.0 from Materialise N.V., Leuven, Belgium.

² Pro/ENGINEER version 2000i from Parametric Technology Corporation, MA, USA.

There are four useful tools in the Designer's Toolbox:

1. Parametric Library

As a first step, the designer has to select a basic unit cell from the parametric library. The parametric library is a database of basic cellular units and packing configurations. Comparable to an electronic catalogue of building blocks, these units will be the basis of scaffolds. Other choices the user can make include triangulation and presence of nodes at intersections of struts.

2. Sizing Routines

There are two types of sizing controls. The first deals with the dimensions of each unit cell such as the length, breadth and height of the cell. This depends on the type of unit cell selected as some unit cells are of equal lengths in all directions. In that case, the user only need indicate one value. The second type of control concerns the overall dimensions of the scaffold in the x, y and z directions.

3. Automated Algorithm

The inputs entered in the user interface and the CAD files of the scaffolds are linked via the algorithm. The algorithm generates the scaffolds in the specified size and also calculates the necessary output values such as porosity, pore size and surface to volume ratio. These values are displayed in the user interface. Examples of generated scaffolds are shown in Figure 3.2.

Figure 3.2: Samples of polyhedral scaffold structures

4. Slicing Routines

As previously mentioned in Section 2.3, one major consideration in tissue scaffolding is the ingrowth of cells into the inner regions of the scaffold. The slicing module incorporated in CASTS is meant to overcome this problem. This sub module allows the user to slice the patient specific scaffold into thinner layers which can be seeded separately and then assembled together before implantation. The block scaffold is first generated and the user can choose to slice the scaffold into different layers (Figure 3.3). There are also orientation features to aid as position markers during re-assembling so that the original shape is retained.

Figure 3.3: (a) Rectangular scaffold block model (b) Layer geometry with orientation features (c) Sliced scaffold model with orientation features

5. Database

While the previous tools allow the user to choose and specify the design parameters, it is also necessary to obtain critical information from CASTS such as pore sizes, surface area-to-volume ratio, and porosity of the scaffold. These values are calculated by the program based on the dimensions of the scaffold as entered by the user. Data will also be collated to correlate the materials used, mechanical properties of the scaffolds, pore size, shapes and porosity, and their interrelationship with the proliferation and the differentiation rate of different cell types. All these information will be stored and reflected in CASTS to be made available to users.

6. Output Interface

The generated CAD files have to be exported in a format which is readable by the fabrication system. For SLS, the standard format is the STL format.. The user may wish to use other interfaces for purposes such as documentation or further manipulation of the models.

3.5 Output Module

Upon completion of the first stage when the scaffold block is generated, the output is in the PRT format or part file in the Pro/ENGINEER interface. The IGES file from the Input Module is then used to change the scaffold to a patient specific shape.

As the main purpose of CASTS is scaffold fabrication, the final output is, by default, a STL file. The STL format is an ASCII or binary file used in manufacturing. It is basically a series of triangular surfaces, each represented by 3 vertices and a normal in Cartesian coordinates, that describes a computer generated solid model.

IV PARAMETRIC LIBRARY & AUTOMATIC ALGORITHM

As discussed in Chapter 2, tissue engineers have long thought that porous structures are the basis for TE scaffolds. Zeltinger *et al* recognised that there was a need to characterise the features of scaffolds such as porosity, pore size, and shape, polymeric composition and overall architecture to observe the effects of these factors on cell growth, proliferation, and differentiation [129]. However, there lacks a systematic database which is required to do such a comparison. Hence, in this project, a first step is taken to study geometric shapes and compile them into a parametric library suitable for designing scaffolds. This library, used in conjunction with an automated algorithm and solid freeform fabrication techniques, can facilitate the compilation of such a database.

4.1 Geometry as a Basis for Structure

4.1.1 Cellular and Non-cellular Solids

Cellular solids

Cellular solids are clusters of cells that are joined through an interconnected network of solid struts or thin walls without any gaps in between the cells. Examples of naturally occurring cellular solids include wood and cancellous bone which are natural structural supports. In the industry, they are commonly used as shock absorbers and in beams. There are two types of cellular solids, open cells and closed cells (see Figure 4.1) [93]. Many conventional techniques of scaffold fabrication such as particulate leaching methods are aimed at producing structures similar to open cellular solids.

Figure 4.1: Examples of (a) open cells and (b) closed cells [93]

Non-cellular solids

Such cells leave small but regular 3-D gaps when they are assembled. Therefore, in an assembly, two pore sizes are obtained - one that is enclosed by the cell itself and another that is enclosed by the gap [27]. The difference between cellular and non-cellular solids is illustrated in Figure 4.2.

Figure 4.2: Examples of (a) A cellular solid - an assembly of rhombic dodecahedron
(b) A non-cellular solid - an assembly of cuboctahedrons (circle indicates gap)

4.1.2 Structure in Nature: Polyhedra

Polyhedral forms are what make up cellular and non-cellular solids. In space, a line can be regarded as a succession of points. The line can be moved in three ‘ways’ to form a polygon, which are two-dimensional shapes of continuously linked segments. The simplest polygon is a triangle with the other possible basic shapes being the square and the circle [130]. Regular polygons that are not self-intersecting are termed convex polygons (Figure 4.3) [131].

Figure 4.3: Five sided regular polygons (*left*) non-self-intersecting (*right*) self-intersecting

A polyhedron is defined as a three-dimensional object bounded by polygons with each edge shared by exactly two polygons [131]. When each of the three basic polygons extends into three-dimensions, they form solids: the triangle results in a tetrahedron, the rectangle in a rectangular block and the circle in a sphere. As the tetrahedron is the most basic of solids, it is known as the primary solid (Figure 4.4) [130].

Figure 4.4: (a) A tetrahedron and (b) its fold-up pattern in 2D

Polyhedral shapes can generally be grouped into two categories: convex and non-convex polyhedra (Figure 4.5). A convex polyhedron contains no holes or indentations. As non-convex polyhedrons have highly complex shapes, they are difficult to model in a CAD (Computer Aided Design) environment and may not be feasible to be fabricated by RP techniques. As a result, they are not considered for the database.

Figure 4.5: Examples of (a) a convex polyhedron - cube and (b) a non-convex polyhedron – stellated truncated hexahedron [131]

Convex polyhedrons can be further classified into four main categories: Platonic solids, Archimedean solids, prisms, and antiprisms, as well as Archimedean duals [131].

Platonic Solids

Also known as the regular solids, Platonic solids are made up of congruent regular polygons. Platonic Solids are governed by Euler's Law [27]:

$$-C + F - E + V = 1 \quad (4.1)$$

where E is the number of edges, V the number of vertices, F the number of faces and C the number of cells of a polyhedron.

There are five platonic solids: the cube, dodecahedron, icosahedron, octahedron, and tetrahedron [130, 132]. Platonic solids have inspheres i.e. spheres inscribed within them that touch all the internal faces of the solid (Figure 4.6).

Figure 4.6: Inspheres of platonic solids [132]

Archimedean Solids

Archimedean solids are made up of two or more convex regular polygons [131, 132].

They satisfy the following equation [132]:

$$(2\pi - \sigma) V = 4\pi \quad (4.2)$$

where σ is the sum of face-angles at a vertex and V is the number of vertices

Also known as the semi-regular solids, Archimedean solids can be generated from Platonic Solids by carrying out one of the following operations: truncation of the corners, expansion, and performing a snub which consists of moving faces of the polyhedra outward and filling the gaps with equilateral triangles [130, 132]. There exist a total of thirteen Archimedean solids, and similar to Platonic solids, they have inspheres [132].

Prisms and Antiprisms

Prisms and Anti-prisms are classified under Archimedean solids but listed separately since they form an infinite series. In general, a prism is a polyhedron that consists of two parallel congruent polygon faces which are connected by parallelograms such as rectangles. An anti-prism also consists of two congruent faces. However, in an antiprism, the two faces are twisted in relation to each other such that while they are parallel, they can only be connected through a series of alternately up and down triangles [131].

Archimedean Duals

For every polyhedron, there is another polyhedron in which the faces and vertices of the polyhedron occupy complimentary positions. This is termed reciprocation and the reciprocal polyhedrons are known as duals. As such, Archimedean duals are duals of Archimedean solids. They are also known as Catalan Solids [132]. In Archimedean duals, all the faces are identical but there are two or more types of vertices. This is because these polyhedra are reciprocals of Archimedean solids which have one or more regular polygons but the same type of vertex. [131].

There are other polyhedra which are also made up of regular faces and convex in nature but do not fall into any of the above categories. These polyhedra are named Johnson

Solids. Most Johnson solids are assemblages of regular polyhedra [131]. Among Johnson solids, only the square pyramid can be repeated regularly in space. Six square pyramids can be joined together to form a cube if the height, h , of the pyramid is half that of the length, l , of its base (Figure 4.7).

Figure 4.7: The squared pyramid (left) and the assemblage of six square pyramids (right)

4.2 Geometric Considerations

4.2.1 Tessellation

One consideration for part fabrication in RP systems is tessellation. In RP systems, the edges of all objects are tessellated [68]. A tessellation is a mosaic of joined polygons (two dimensions) or polyhedra (three dimensions) [132]. Following the concept of closest packing, a sphere has the least surface to volume ratio and in a two-dimensional representation; a circle encloses the largest amount of space for a given length of parameter. However, when patterns of closest packed circles are examined, there are pockets of gap in between the circles. These cannot be read by the software of RP systems. As a result, a tessellation is carried out to approximate the geometry [133]. Figure 4.8 shows the change in geometry as circles are tessellated.

Figure 4.8: Changing closest packed circles into closest packed hexagons

A regular polygon has 3 or more sides and angles, all of which are equal. When a tessellation is made up of congruent regular polygons, it is called a regular tessellation. There are only three such tessellations: tiling of triangles, squares, and hexagons. If, however, the tessellation consists of two or more types of polygons, they are called semiregular or Archimedean tessellations. Examples of such tessellations are shown in Figure 4.9.

Figure 4.9: Examples of regular tessellations (a) (b) (c) and semiregular tessellations (d) (e)

4.2.2 Triangulation

When equal-sized spheres are stacked together on top of each other, two types of formations are possible. The first, as shown in Figure 4.10(a), is the rectangular formation. It is easily seen that such a formation is unstable and it can easily collapse. However, if the spheres were to be arranged in a triangular formation such as that seen in Figure 4.10(b), the spheres will remain in position. This is because in this formation, the spheres exist in their lowest energy state [133]. This also applies in the 3-D packing of equal-sized spheres and the packing is most dense when it is triangulated.

(a)

(b)

Figure 4.10: (a) Rectangular stacking of equal sized spheres and (b) Triangular stacking of equal sized spheres

It is evident that to be geometrically stable, all shapes need to be triangulated. Figure 4.11 shows the examples of triangulated polyhedrons. In the case of cubes, cubes formed by stacking 8 spheres (one at each corner) create an unstable condition. To form a stable cube (seen in Figure 4.11(h)), at least 14 closest packed equal sized spheres are required. It is easily seen that if a diagonal is drawn across one face of the cube, it forms two triangles.

Figure 4.11: Two and three-dimensional views of figures formed by closest packed equal spheres
 (a)-(b) Square Pyramid (c)-(d) Octahedron (e)-(f) Cuboctahedron (g)-(h) Cube

4.3 Space Filling Systems

4.3.1 Background

Space filling systems are required to allow repetition of polyhedral shapes in space. In 3-D space, there are two main types of space filling systems: unary and multiple systems. They are classified according to the number of types of polyhedron that each system requires; a unary system consists of only 1 type of polyhedron whereas multiple systems range from combinations of 2 (binary systems) to 4 types of polyhedron (quaternary systems) [133].

There are a few basic rules for space filling systems [133].

1. The different polyhedrons must have matching faces in common i.e. there must be some symmetry between adjacent polyhedra.
2. The vertices of the array must be congruent.
3. In joining of polyhedra, dihedral angles formed by faces meeting around a common edge must add up to 360° . This ensures that all space is occupied without the intersection of contiguous cells. The dihedral angle refers to the angle formed between the planes of two adjacent polygons. Figure 4.12 illustrates the dihedral angle formed between two planes.

Figure 4.12: Illustration of dihedral angle

Table 4.1 lists 24 possible space filling systems of convex polyhedra.

Table 4.1: Unary and Multiple space filling systems [133]

Item	Space Filling System	Space Filling Ratio	Polyhedron Class
1	Square prism/Cube	-	Prisms and anti prisms/ Platonic solids
2	Truncated Octahedron	-	Archimedean solids
3	Triangular prism	-	Prisms and anti prisms
4	Hexagonal prism	-	Prisms and anti prisms
5	Rhombic dodecahedron	-	Archimedean duals
6	Square pyramid	-	Johnson solids
7	Tetrahedron Octahedron	2 : 1	Platonic solids Platonic solids
8	Tetrahedron Truncated tetrahedron	1 : 1	Platonic solids Archimedean solids
9	Octahedron Truncated Cube	1 : 1	Platonic solids Archimedean solids
10	Octahedron Cuboctahedron	1 : 1	Platonic solids Archimedean solids
11	Truncated cuboctahedron Octagonal prism	1 : 3	Archimedean solids Prisms and anti prisms
12	Octagonal prism Cube	1 : 1	Prisms and anti prisms Platonic solids
13	Triangular prism Hexagonal prism	2 : 1	Prisms and anti prisms Prisms and anti prisms
14	Triangular prism Hexagonal prism	8 : 1	Prisms and anti prisms Prisms and anti prisms
15	Triangular prism Cube	2 : 1	Prisms and anti prisms Platonic solids
16	Triangular prism Twelve-prism	2 : 1	Prisms and anti prisms Prisms and anti prisms
17	Truncated cuboctahedron Truncated octahedron Cube	1 : 1 : 3	Archimedean solids Archimedean solids Platonic solids
18	Truncated cuboctahedron Truncated cube Truncated tetrahedron	1 : 1 : 2	Archimedean solids Archimedean solids Archimedean solids
19	Rhombicuboctahedron Cuboctahedron Cube	1 : 1 : 3	Archimedean solids Archimedean solids Platonic solids
20	Rhombicuboctahedron Cube Tetrahedron	1 : 1 : 2	Archimedean solids Platonic solids Platonic solids
21	Truncated octahedron Cuboctahedron Truncated tetrahedron	1 : 1 : 2	Archimedean solids Archimedean solids Archimedean solids
22	Hexagonal prism Triangular prism Cube	1 : 2 : 3	Prisms and anti prisms Prisms and anti prisms Platonic solids
23	Hexagonal prism Twelve-prism Cube	2 : 1 : 3	Prisms and anti prisms Prisms and anti prisms Platonic solids
24	Rhombicuboctahedron Truncated cube Octagonal prism Cube	1 : 1 : 3 : 3	Archimedean solids Archimedean solids Prisms and anti prisms Platonic solids

4.3.2 Selection Criteria of Polyhedra

Not all of the space filling systems listed in Table 4.1 may be deemed feasible in the context of tissue scaffold design. For instance, a space filling system may contain a polyhedron with a large number of edges which, when triangulated, will result in small pore sizes and also decrease overall porosity. In considering the suitable space filling systems, many factors have to be considered such as mechanical properties as well as complexity of modelling and generating of algorithm, which in turn affect the amount of resources required. A final selection is made to choose suitable polyhedra systems which satisfy the following criteria:

1. Polyhedrons that have faces with a number of edges greater than eight are avoided. This is because the increase in number of edges in a face will decrease the ability of vertices to resist normally applied concentrated loads. Furthermore, there is a tendency for pore size to drop when face triangulation is introduced for additional mechanical strength.
2. In order to reduce the complexity of modelling and generating algorithm, only binary systems are considered.
3. Systems that require four or more layers to repeat in any orientation should be avoided.

These rules limit the type of polyhedra that are available to form space filling systems. In Figure 4.13, a binary space filling system is obtained by arranging octahedron (the lighter solid) and tetrahedron cells (the dark solids) in the ratio of 1:2.

Figure 4.13: Octahedron-Tetrahedron space filling system

Selection Results

Each of the space filling systems listed in Table 4.1 is analysed individually to determine if it meets the selection criteria. A summary of the selection process and the selected space filling systems are shown in Table 4.2 and Table 4.3 respectively.

Table 4.2: Preliminary selection results of space filling systems

Item	Space Filling System	Selection Criteria			Selected?
		1	2	3	
1	Square prism/Cube	Pass	Pass	Pass	Yes
2	Truncated Octahedron	Pass	Pass	Pass	Yes
3	Triangular prism	Pass	Pass	Pass	Yes
4	Hexagonal prism	Pass	Pass	Pass	Yes
5	Rhombic dodecahedron	Pass	Pass	Pass	Yes
6	Square pyramid	Pass	Pass	Pass	Yes
7	Tetrahedron Octahedron	Pass	Pass	Pass	Yes
8	Tetrahedron Truncated tetrahedron	Pass	Pass	Fail	No
9	Octahedron Truncated Cube	Pass	Pass	Pass	Yes
10	Octahedron Cuboctahedron	Pass	Pass	Pass	Yes
11	Truncated cuboctahedron Octagonal prism	Pass	Pass	Pass	Yes
12	Octagonal prism Cube	Pass	Pass	Pass	Yes
13	Triangular prism Hexagonal prism	Pass	Pass	Pass	Yes
14	Triangular prism Hexagonal prism	Pass	Fail	Pass	No
15	Triangular prism Cube	Pass	Pass	Pass	Yes
16	Triangular prism Twelve-prism	Fail	Pass	Pass	No
17	Truncated cuboctahedron Truncated octahedron Cube	Pass	Fail	Pass	No
18	Truncated cuboctahedron Truncated cube Truncated tetrahedron	Pass	Fail	Pass	No
19	Rhombicuboctahedron Cuboctahedron Cube	Pass	Fail	Pass	No
20	Rhombicuboctahedron Cube Tetrahedron	Pass	Fail	Pass	No
21	Truncated octahedron Cuboctahedron Truncated tetrahedron	Pass	Fail	Pass	No
22	Hexagonal prism Triangular prism Cube	Pass	Fail	Pass	No
23	Hexagonal prism Twelve-prism Cube	Fail	Fail	Pass	No
24	Rhombicuboctahedron Truncated cube Octagonal prism Cube	Pass	Fail	Pass	No

Table 4.3: Selected space filling systems

Item	Space Filling System	Space Filling Ratio	Polyhedron Class
1	Tetrahedron Octahedron	2 : 1	Platonic solids Platonic solids
2	Octahedron Truncated Cube	1 : 1	Platonic solids Archimedean solids
3	Triangular prism	-	Prisms and anti prisms
4	Square prism/Cube	-	Prisms and anti prisms/ Platonic solids
5	Square pyramid	-	Johnson solids
6	Rhombic dodecahedron	-	Archimedean duals
7	Truncated Octahedron	-	Archimedean solids
8	Hexagonal prism	-	Prisms and anti prisms
9	Octahedron Truncated Cube	1 : 1	Platonic solids Archimedean solids
10	Truncated cuboctahedron Octagonal prism	1 : 3	Archimedean solids Prisms and anti prisms
11	Octagonal prism Cube	1 : 1	Prisms and anti prisms Platonic solids
12	Triangular prism Hexagonal prism	2 : 1	Prisms and anti prisms Prisms and anti prisms
13	Triangular prism Cube	2 : 1	Prisms and anti prisms Platonic solids

4.4 Determination of Control Dimensions

The cells are modelled in CAD environment for visualisation and further evaluation. As the unit matrices are meant for use as tissue engineering scaffolds, open cells are required which only consist of struts that connect each face to the next. The cells are modelled such that by the shape of the cell can be controlled by varying a minimum number of parameters. The number of parameters is decided according to the nature of the shape. For prisms, a minimum of 3 parameters is required to control the dimensions: length and thickness of the strut as well as the height of the unit cell. For the remaining shapes, length and thickness of the strut are sufficient as the length of the strut itself decides the height of the unit cell [27]. Figure 4.14 shows the control dimensions used in a triangular prism.

The open unit cells are modelled in Pro/ENGINEER through the following steps. (Note: the sections of the struts were first modelled as triangles.)

1. The closed cell structure of all cells is first modelled.
2. Cuts are made via planes perpendicular to each surface to create shells.
3. More cuts are made to ensure that the struts are isosceles triangles.

Figure 4.14: Control dimensions of a triangular prism

Table 4.4 shows eleven of the polyhedral shapes that have been modelled.

Table 4.4: CAD models of polyhedral units

Sample	Polyhedron Category	Classification	Control Dimensions	No. of faces
Prisms	Cellular	l, h, t	5
l, h, t	8
l, h, t	10
Prisms/ Platonic Solids		l, h, t	6
N.A Johnson Solid		$l, t (h = l/2)$	5
Archimedean Solids		l, t	14
l, t	14
Non-cellular	l, t	14
l, t	26
l, t		26
Archimedean Duals		l, t	12

Note: l = length of strut , h = height of strut, t = thickness of strut

4.5 The Parametric Library

Strut design and control dimensions

After making the final selection of the polyhedral shapes, the control dimensions are re-examined to validate the application. Three basic geometric shapes, namely, the cube, the triangular prism, and the cylinder, are considered for use as struts interconnecting the scaffold structures. It is found, by simple calculation, that the cylinder has the largest area to volume ratio (which aids cell proliferation) and as such, it is chosen as the default geometry for struts. The cylinder also has the added advantage in that the curved surfaces will help to minimise stress concentration under loads.

Hence, the thickness, t , of the strut is now represented by the radius, r . The definitions of the length, l , of the strut as well as the height, h , of the unit matrices remain. An additional dimension is added for the triangular prism, such that when the base area is not an equilateral triangle, the breadth, b , is independently defined.

Node

When the unit cells are assembled, there will be intersection points where the struts meet. These points of intersection create sharp corners and regions of stress concentration (see Figure 4.15a). To minimise this problem, a node is introduced at such junctions (see Figure 4.15b). To be effective, the node must be of a minimum diameter for a given number of struts at a specific diameter of strut.

$$R = 2 \times \frac{r}{\sin \left[\frac{\tan^{-1} \left(\frac{b}{l} \right)}{2} \right]} \quad (4.3)$$

where R is the maximum value of a function of any two joined struts of radius r in a unit cell of breadth b and length l .

Figure 4.15: Intersection of struts (a) without node (b) with node

Unit matrices

There are 3 main steps in modelling the unit matrices:

1. Creating the node,
2. Creating the negative mould model, and
3. Generating the polyhedron model.

1. First, the node is modelled in Pro/ENGINEER, with its origin at the centre of geometry, as shown in Figure 4.16 and saved as a part.

Figure 4.16: A node

- 2a. To create the negative mould, the closed cell of the polyhedron is first modelled, again, with its origin at the centre of gravity. A closed cell of octahedron is shown in Figure 4.17.

Figure 4.17: A closed cell of octahedron

- 2b. Next, sets of coordinate systems are created, one for each vertex of the polyhedron as illustrated in Figure 4.18. There are now a total of 7 coordinate systems altogether including the one at the centre of geometry. These coordinate systems are to aid in aligning of models for Boolean operations.

Figure 4.18: Closed cell of octahedron with a co-ordinate system at each vertex

- 2c. The first cut is made along one of the edges such that it runs along the entire edge. The cross-section of the cut is shown in Figure 4.19a. More cuts are created either through duplicating or constructing the feature until all edges have similar cuts. The resulting polyhedron is shown in Figure 4.19b.

Figure 4.19: (a) Cross-section of cut along each edge (b) Octahedron model after cuts along all edges

- 2d. The nodes are assembled onto each vertex by using the *External Copy Geometry* function. In assembling, the coordinate system of each node is aligned to the coordinate system at a vertex of the polyhedron model as shown in Figure 4.20.

Figure 4.20: Octahedron model with nodes attached at vertices

- 2e. A cut is performed with reference to the copied external geometry of the node such that the vertex now has a hollow area in the shape of a segment of the sphere. This is repeated until all the vertexes assume similar shapes. The completed negative mould of the octahedron is displayed in Figure 4.21.

Figure 4.21: Completed negative mould of the octahedron

- 3a. To obtain the open cell, the closed cell is first modelled as in step 2a.
- 3b. The *External Copy Geometry* function is again used to copy the imprint of the negative mould onto the closed cell. The two cells are mapped together by aligning the coordinate systems at the centre of geometry of each cell. In Figure

4.22, the lighter-coloured cell is the closed cell and the darker-coloured cell is the negative mould.

Figure 4.22: Assembly of the closed cell and negative mould of octahedron

3c. A Boolean operation is performed which leaves with cell with open interconnected struts (see Figure 4.23).

Figure 4.23: The open cell of octahedron

Figure 4.24 shows four examples of the open cells of different matrices and their negative mould.

Figure 4.24: Selected polyhedral unit cells with major control parameters (a) octahedron, (b) tetrahedron, (c) triangular prism, (d) square prism and (e) – (h) are the negative cell structures for (a) – (d) respectively [134]

In order to automate the assembly, the unit matrices must be in repeatable units such that they can be duplicated. It is noted that while configurations 2-8 are based on repeatable units, other configurations such as configuration 1 need to be modelled to combine the 2 polyhedrons that together act as repeating units. The combined geometry is modelled in the same way as the unit matrices. Figure 4.25 shows the completed octahedron-tetrahedron basic unit.

(a)

(b)

Figure 4.25: (a) Open cellular unit and (b) the negative mould of octahedron-tetrahedron

4.6 Development of a Scaffold Algorithm

4.6.1 Critical Scaffold Parameters

Before programming for the scaffold generation, it is important to examine the important parameters of the scaffold. The following parameters are deemed critical and are formulated into the scaffold library program.

Pore size

Pore size is defined as the largest sphere that can be contained within a 3-D space. In TE scaffolds, too small a pore size will prevent the cells from proliferating inwards and can also cause occlusion. However, if pore sizes are too large, the cells will slip through the pores and grow at the bottom surface instead. In two-dimension, pore size is dictated by the largest circle that can be inscribed within a polygonal face of a unit cell. In the parametric library, the faces of cellular units are composed of triangles, rectangles, hexagons and octagons. As illustrated in Figure 4.26, except for triangular faces, the largest circle that can be inscribed inside the face is bounded by parallel side edges with the minimum tangential distance between them. For a triangular face, the radius of the largest circle is the tangential distance from the intersection point of the three angle bisectors to the side edge.

Figure 4.26: Illustration of largest circle in face of (a) rectangular (b) hexagonal (c) octagonal (d) triangular cellular units.

With reference to Figure 4.27, the general expression for diameter of the largest circle inscribed inside an arbitrary triangle can be found by trigonometry to be:

$$\text{Diameter of largest circle, } d = 2 \times \frac{l \times \sin a \times \sin b}{\sin c} \quad (4.4)$$

where l = length of the cellular unit and a, b and c are angular dimensions as defined in Figure 4.27.

Figure 4.27: Largest circle inscribed inside an arbitrary triangle

Unary systems will have only one pore size since the 3-D space within each cell is uniform. However, for binary systems, there will be 2 different pore sizes resulting from the space within the cell itself (maximum pore size) and also the gaps in between the cells (minimum pore size). In each case, the pore size is determined by placing the centre of a imaginary sphere to coincide with the centre of gravity of the space, and increasing the diameter until the sphere becomes tangent to one of the circumscribing faces of the cell [135]. Figure 4.28 illustrates with circles indicating the pore sizes.

Figure 4.28: Definition of pore size [134]

Surface to volume ratio

Anchorage-dependent cells are known to proliferate along surfaces. As a result, a high surface to volume ratio means that there is more space for the tissue to attach and grow. As such, the surface to volume ratio is a measure of the effectiveness of the scaffold. The surface area to volume ratio is calculated by dividing the generated surface area value by the total volume of the assembly.

To reflect this parameter in the layout requires mathematical derivations of surface area and cell volume.

The general expression for calculating surface area of a scaffold with nodes is

$$\text{Surface Area of Scaffold} = \sum_{w=1} y_w \left\{ \sum_{i=1} A_i m_i + \sum_{j=1} B_j n_j \right\}_w \quad (4.5)$$

where A is the surface area of strut segment of a particular length in the unit cell type,

B is the surface area of a truncated node segment in the unit cell type,

m, n are the quantities of strut segment and truncated node segment in the unit cell type respectively,

w is the unit cell type in the scaffold,

y is the quantity of the unit cell type in the scaffold.

Similarly, a general expression for volume of a scaffold with nodes is given by

$$\text{Volume of Scaffold} = \sum_{w=1} y_w \left\{ \sum_{i=1} C_i m_i + \sum_{j=1} D_j n_j \right\}_w \quad (4.6)$$

where C is the volume of strut segment of a particular length in the unit cell type,

D is the volume of a truncated node segment in the unit cell type,

and the other variables are as previously defined.

Porosity

Another important factor to be determined is the relative density of the scaffold which is calculated as shown in Equation 4.4.

$$\text{Relative density} = \frac{\rho^*}{\rho_s} \quad (4.7)$$

where ρ^* is the density of the cellular solid and ρ_s is the density of the material

The macroporosity value can be related directly to the ratio of density of the scaffold, ρ^* , and the density of the solid polyhedron model, ρ_s , (i.e. the relative density of the scaffold). However, when the mass value of both are identical as in this case, the porosity value can be related to the volume of the scaffold structure, V^* , and the volume of the solid polyhedron, V_s .

The strength of the structure depends on its relative density while the porosity is directly related to the mechanical strength of the scaffold. This is because porosity is a measure of the amount of material hollowed out from the solid volume. Studies carried out on interconnected open-cell structures show that the porosity of such structures has a minimum value of 70% [93]. This makes it suitable for their application as TE scaffolds which need porous interconnected networks for exchange of nutrients and wastes to and from the cells.

4.6.2 Mathematical solutions: Octahedron-Tetrahedron space filling system

In this section, a mathematical solution for the critical parameters is presented for one of the cellular units of the parametric library, the octahedron-tetrahedron space filling system. Figure 4.29(a) shows the two unit cells that make up the octahedron-tetrahedron dual space filling system. In this system, each octahedron unit cell is joined to two units of tetrahedrons. The resulting system is shown in Figure 4.29(b). In Figure 4.29(a), three types of struts have been labelled. For this configuration, there is six of each per unit cell. The following calculations are based on a unit cell with these dimensions: cell length, l ; strut radius, r and node radius, R .

Figure 4.29: (a) Octahedron and Tetrahedron polyhedra (b) Octahedron-Tetrahedron configuration with 1:2 ratio

Pore Size

As a binary system, there are two pore sizes for this configuration. The first is confined by a set of equilateral triangles. Using Equation (4.4), for a unit cell with cell length l , the pore size is given by:

$$\text{Pore size} = \left[\frac{l}{\sqrt{2}} - 2 \left(\frac{r}{\tan 30} \right) \right] \tan 30 \quad (4.8a)$$

The second is calculated as:

$$\text{Pore size} = \frac{\frac{l}{\sqrt{2}} - \frac{r}{\tan(45)} - \frac{r}{\tan(22.5)}}{\sin(112.5) \cdot \sin(135)} \quad (4.8b)$$

Surface area to volume ratio

In order to calculate the surface area to volume ratio, the lengths of the struts are formulated first. From Figure 4.29,

$$\text{Length of strut 1} = l - 2 \left[R - \frac{r}{\tan \left(45 + \frac{1}{2} \cos^{-1} \left(\frac{r}{R} \right) \right)} \right] \quad (4.9)$$

$$\text{Length of strut 2} = \text{Length of strut 1} \quad (4.10)$$

$$\text{Length of strut 3} = \text{Length of strut 1} \quad (4.11)$$

Both equations (4.12) and (4.13) are obtained from Figure 4.29.

From the lengths, the surface area of each strut can be calculated as:

$$A_l = \text{Surface area of strut 1} = \frac{2\pi r}{360} \left[\cos^{-1} \left(\frac{1}{3} \right) \right] \left\{ l - 2 \left[R - \frac{r}{\tan \left(45 + \frac{1}{2} \cos^{-1} \left(\frac{r}{R} \right) \right)} \right] \right\} \quad (4.12)$$

$$A_2 = \text{Surface area of strut 2} = \frac{2\pi r}{360} \left[\cos^{-1} \left(-\frac{1}{3} \right) \right] \left\{ l - 2 \left[R - \frac{r}{\tan \left(45 + \frac{1}{2} \cos^{-1} \left(\frac{r}{R} \right) \right)} \right] \right\} \quad (4.13)$$

$$\begin{aligned} A_3 = \text{Surface area of strut 3} &= A_1 + A_2 \\ &= \pi r \left\{ l - 2 \left[R - \frac{r}{\tan \left(45 + \frac{1}{2} \cos^{-1} \left(\frac{r}{R} \right) \right)} \right] \right\} \end{aligned} \quad (4.14)$$

The surface area of the node, A_4 , can be calculated as:

$$A_4$$

=

$$\begin{aligned} &4\pi R^2 - 2 \left[\frac{1}{360} \left[\cos^{-1} \left(\frac{1}{3} \right) \right] (6) + \frac{1}{360} \left[\cos^{-1} \left(-\frac{1}{3} \right) \right] (6) + \frac{1}{2} (6) \right] (2\pi R) \left[\frac{r}{\tan \left(45 + \frac{1}{2} \cos^{-1} \left(\frac{r}{R} \right) \right)} \right] \\ &= 4\pi R^2 - 24\pi R \left\{ \frac{r}{\tan \left(45 + \frac{1}{2} \cos^{-1} \left(\frac{r}{R} \right) \right)} \right\} \end{aligned} \quad (4.15)$$

Hence, for a scaffold with N number of cells, total surface area of the scaffold is given by:

$$\text{Total surface area} = N (6(A_1 + A_2 + A_3) + A_4) \quad (4.16)$$

The volume occupied by the struts and the node is calculated individually.

$$V_1 = \text{Volume of strut 1} = \frac{1}{360} (\pi r^2) \left[\cos^{-1} \left(\frac{1}{3} \right) \right] \left\{ l - 2 \left[R - \frac{r}{\tan \left(45 + \frac{1}{2} \cos^{-1} \left(\frac{r}{R} \right) \right)} \right] \right\} \quad (4.17)$$

$V_2 = \text{Volume of strut 2}$

$$= \frac{1}{360} (\pi r^2) \left[\cos^{-1} \left(-\frac{1}{3} \right) \right] \left\{ l - 2 \left[R - \frac{r}{\tan \left(45 + \frac{1}{2} \cos^{-1} \left(\frac{r}{R} \right) \right)} \right] \right\} \quad (4.18)$$

$V_3 = \text{Volume of strut 3} = \text{Volume of strut 1} + \text{volume of strut 2}$

$$= (\pi r^2) \left\{ l - 2 \left[R - \frac{r}{\tan \left(45 + \frac{1}{2} \cos^{-1} \left(\frac{r}{R} \right) \right)} \right] \right\} \quad (4.19)$$

The node volume, V_4 , is calculated as:

V_4

$$= \frac{4}{3} \pi r^3 - 12 \left\{ \frac{1}{3} \pi \left[\frac{r}{\tan \left(45 + \frac{1}{2} \cos^{-1} \left(\frac{r}{R} \right) \right)} \right]^2 \right\} \left\{ 3R - \left[\frac{r}{\tan \left(45 + \frac{1}{2} \cos^{-1} \left(\frac{r}{R} \right) \right)} \right] \right\} \quad (4.20)$$

Hence, total effective volume = $N (V_1 + V_2 + V_3 + V_4)$ (4.21)

Therefore, surface area to volume ratio = $\frac{\text{Total surface area}}{\text{Total effective volume}}$

$$= \frac{6(A_1 + A_2 + A_3) + A_4}{V_1 + V_2 + V_3 + V_4} \quad (4.22)$$

Porosity

From equation (4.21), the total effective volume is $N (V_1 + V_2 + V_3 + V_4)$. The solid volume of the unit cell is equivalent to the sum of the volume of one octahedron closed cell and two tetrahedron closed cells.

$$\text{Volume of octahedron} = 2 \left(\frac{1}{3} \right) \left(\frac{l}{\sqrt{2}} \right) l^2 = \frac{\sqrt{2}}{3} l^3 \quad (4.23)$$

$$\text{Volume of tetrahedron} = \left(\frac{l}{\sqrt{2}} \right)^3 - \left(\frac{1}{3} \right) \left(\frac{l}{\sqrt{2}} \right) l^2 = \frac{l^3}{6\sqrt{2}} \quad (4.24)$$

V , total volume of solid is given by:

$$V = N \left(\frac{\sqrt{2}}{3} l^3 \right) + 2N \left(\frac{l^3}{6\sqrt{2}} \right) = \frac{1}{\sqrt{2}} N l^3 \quad (4.25)$$

Hence, porosity of the scaffold model is:

$$\begin{aligned} \text{Porosity} &= 1 - \frac{\text{Total volume}}{\text{Total effective volume}} \\ &= 1 - \frac{N (V_1 + V_2 + V_3 + V_4)}{V} \end{aligned} \quad (4.26)$$

4.6.3 Generation of patient-specific scaffold

Means of assembly

Previous experience [135] has shown that when an assembly of structures is built in SLS, the software reads the units of the assembly as separate components and hence,

triangulation is done separately for each unit. Figure 4.30 shows an assembly of two square prism unit cells. The dotted lines are the edges of triangular facets across the surface of the assembly. As the inset of Figure 4.30 shows, the cells do not share any facets. This affects the quality of the part. The absence of continuous triangular facets across the boundary of the cells causes the laser to skip the boundaries of the unit cells and weakens the bonding strength between each unit cell.

Figure 4.30: Separate triangular facets at the boundary of assembled units

As a result, to improve scaffold fabrication, the software must interpret the assembly as one unit rather than a group of separate units. Here, reverse engineering is used. One common way of forming cellular solids from polymers is to introduce gas bubbles into the liquid polymer and to allow the bubbles to enlarge and eventually stabilise. This is done by controlling the pressure of the gas. Next, the entire structure is solidified by cross-linking or cooling [98]. The scaffold algorithm is created using the same principle. Instead of bubbles, the negative mould of the scaffold is employed. By subtracting this

from a block of material, one can obtain the scaffold structure with user-defined architecture [100].

Mode of assembly

As mentioned earlier, the negative models of the cells are first assembled to obtain the negative mould which is then subtracted from a block of material via Boolean operations to obtain the open porous network of cellular units. The concept is to first place a unit cell and add on repeating units to obtain the required dimensions. In Figure 4.31, the darker cell is the starting unit and the lighter cells are the add-on units. Only one cell in each direction is shown for clarity; in the program, the assembly will resemble a block.

Figure 4.31: Mode of assembly

The negative mould assembly is built by carrying out the following steps:

1. The distance between each assembled unit cell in a given direction is termed the pitch. This pitch is defined in relation to the user-defined parameters of the cell and entered into the program listing. The pitch is measured from the origin (centre of gravity) of each unit to the next, and varies according to the type of cell as well as the direction of the co-ordinate system as shown in Figure 4.32.

Figure 4.32: Assembly of Square Pyramids

2. Next, a co-ordinate system is created by relating the values of the pitch to its placement. The number of co-ordinate systems thus created depends on the number of cells required in each direction (calculated based on user input).
3. The unit cells are then assembled by mapping their origins onto the co-ordinate systems created in Step 2 (see Figure 4.33).

Figure 4.33: The assembled virtual mould [100]

4. The block of material is created next. If necessary, the program performs a compensation to the dimensions specified by the user so that the dimensions in each direction are in exact multiples of those of the unit cell. If the values are smaller, they are rounded off to the next multiple. For instance, if a user specifies a value of 15 mm in x-direction for a basic unit cell of length 2mm, the length of the block of material generated is 16mm.
5. Finally, an automated Boolean operation is programmed to subtract the virtual mould from the block of material, leaving a porous network of structures as shown in Figure 4.34.

Figure 4.34: Porous structure with desired internal microarchitecture [100]

4.7 User Interface

The scaffold assembly is linked to the interactive layout via declared global dimensions which define the parameters of the scaffold (see Appendix A for a sample of the layout). There are three types of parameters in the interface. *Primary parameters* control the packing configuration and allow the user to choose if there should be nodes and also whether the packing should be triangulated. *Secondary parameters* control the size of the unit cell as well as the overall size of the scaffold. *System generated parameters* are determined within the algorithm (using the formulations previously programmed) based on the user inputs, and displayed in the interface. Pore size, porosity, and surface to volume ratio are examples of such parameters. Table 4.5 shows the list of important parameters used in the generation of scaffold and Figure 4.35, six of the polyhedral shapes and generated CAD scaffolds.

Table 4.5: Key user-defined parameters and system outputs [100]

Item	Parameter	Type	Description
1	Polyhedrons packing configuration	User-defined	Specified regular or alternate patterning in assembly
2	Name of cellular unit 1		Specified the polyhedral unit cell to be used in the assembly
3	Name of cellular unit 2*		Specifies whether additional polyhedral unit cell to be used (for alternate patterning assemblies only)
4	Triangulation (default = 'on')		Specifies whether triangulation of cells is required
5	Node (default = 'on')		Specifies whether nodes are required
6	Strut type (default = 'circular')		Specifies cross sectional design of struts
7	Overall cell length		Specifies the dimensions of the selected polyhedral unit cell
8	Overall cell breadth *		
9	Overall cell height *		
10	Strut diameter		Specifies the strut diameter
11	Node diameter *		Specifies the node diameter
12	Surface to volume ratio	System generated	Output surface area to volume ratio
13	Porosity		Output porosity value of assembly
14	Maximum pore size		Output maximum pore size in assembly
15	Minimum pore size		Output minimum pore size in assembly
16	Scaffold length resolution		Output the number of cell/unit length in the x-, y-, and z-coordinate directions
17	Scaffold breadth resolution		
18	Scaffold height resolution		
19	Cellular unit 1 quantity		Output the quantity of unit cells used in assembly
20	Cellular unit 2 quantity		

* Optional user-defined parameters depending on the polyhedral unit cell selected

Figure 4.35: Six of the polyhedral shapes in the library and the generated CAD scaffolds that are formed by combining the shapes

4.8 Verification of Parametric Library and Automatic Algorithm

It is important to verify the usefulness and application of the parametric library and the algorithm. As stated in Section 1.2, there are 2 main stages to this. Firstly, it must be verified that the SLS system is capable of building such intricate microarchitecture, and secondly, a biomaterial needs to be used to fabricate viable scaffolds.

Preliminary investigations are also carried out to see the effect of varying parameters of the SLS on the quality of the scaffolds. Once the practicability of SLS as the means of scaffold fabrication is established, biomaterials are incorporated. Figure 4.35 shows the direction of work done. Details are reported in Chapter 5 (step 1) and Chapter 6 (steps 2 and 3).

Figure 4.36: Direction of work done for verification of CASTS

V CASE STUDY – FEMUR IMPLANT

5.1 Generation of Patient Specific Scaffold

As shown earlier in Figure 3.1, the application of CASTS combines the created parametric library and automatic algorithm with imaging technologies to generate patient specific scaffolds. A case study is presented in this chapter. CASTS is used to design and generate a patient specific femur scaffold. This case study is meant to validate CASTS, and also acts as a concept model which can be used as a guide to further fine tune the system.

5.1.1 Generating the scaffold via CASTS

The following steps show how a user-defined scaffold is generated from CASTS.

1. The user enters the Pro/ENGINEER environment and the *Working Directory* of the session is set to CASTS.
2. The file *scaffold.lay* (layout) is retrieved (see Figure 5.1).

Item	Description	Parameter Name	Value	Name/Unit
1	Polymer	unit_cell_type	1	
2	Polymer	unit_cell1_name	Octahedron	
3	Polymer	unit_cell2_name	Tetrahedron	
4	Space	cell_filling_ratio	1:2	
5	Triangulation	triangulation	YES	
6	Process	node	YES	
7	Type of Fused Deposition Modeling (FDM)	RP_process_type	1	SLS
8	Type of material used for building scaffold 1 - PLGA 4 - PGA 7 - HDPE 2 - D,L-PLA 5 - PLC 3 - L-PLA 6 - LDPE	material_type	1	PLGA
9	Type of strut cross-section R - round	strut_type	R	
10	Overall strut length of cellular unit	overall_cell_length	10.000000	mm
11	No input required	overall_cell_breadth	0.000000	mm
12	No input required	overall_cell_height	0.000000	mm
13	Diameter	strut_diameter	1.000000	mm
14	Minimum	minimum_node_diameter	2.000000	mm
15	Diameter	node_diameter	4.000000	mm
16	Overall direction	overall_specimen_length	30.000000	mm
17	Overall direction	overall_specimen_breadth	55.000000	mm
18	Overall direction	overall_specimen_height	40.000000	mm
19	Cell	cell_buffer	1	cells
20	Size	cure_width	0.500	mm
21	Layer	cure_depth	1.000	mm
22	Layer	cure_speed	5.000	mm/s
23	Density	material_density_range	1.270 - 1.340	g/cm ³
24	Density	material_density	1.340000	g/cm ³
25	Dimension direction	usable_scaffold_length	30.000000	mm
26	Dimension direction	usable_scaffold_breadth	51.961524	mm
27	Dimension direction	usable_scaffold_height	48.989705	mm
28	Total surface area of scaffold structure	total_structural_area	27331.534200	mm ²
29	Total volume of scaffold structure	total_structural_volume	10864.406355	mm ³
30	Total volume of space within scaffold structure	total_space_volume	92259.332568	mm ³
31	Surface to volume ratio	surface_volume_ratio	2.516	
32	Porosity	porosity	0.912	
33	Minimum pore size	minimum_pore_size	4.773503	mm
34	Maximum pore size	maximum_pore_size	4.773503	mm

Figure 5.1: User interface of CASTS showing the sequence of input

- Using the *Detail* menu, the primary inputs (coloured red) are entered. Once completed, the layout is regenerated. The primary outputs (orange) and secondary inputs (blue) change with the primary input values. Prompts appear under the Description column to get the user to enter additional information.
- The secondary inputs are modified as prompted by the system and the layout is again regenerated.
- Before proceeding to the next step, the layout is checked for any error messages which are displayed in the Error Status Check column. Any error shown is rectified until the column shows no errors and displays the message: "Proceed with model generation".
- The secondary output values (yellow) are scanned to ensure that the scaffold requirements are met.
- Once completed, *scaffold.asm* (assembly) is retrieved. The assembly is regenerated to reflect the modified inputs in the layout.
- From the *Model Tree* of the scaffold assembly, *scaffold.prt* (part) is retrieved.

5.1.2 Data acquisition and manipulation

Topographic data at the site of defect, where the scaffold is to be implanted, must be known so that the scaffold can be customised for the patient. Such data is necessary for constructing a spatially and anatomically accurate scaffold in CAD that will fit the defect. Under most circumstances, such information may be readily acquired from medical imaging data that was taken to diagnose the patient's condition, or through physical examination and sound knowledge of the human anatomy. With tomographic imaging systems such as CT or MRI, the internal and external tissue structures of the patient would be represented as a series of equally spaced axial 2-D transverse tomograms and it is possible to attain accurate measurements of the defect.

The following steps show how the CT data is arranged and interpreted into a format that is readable by Pro/ENGINEER so that the final scaffold obtained is in the shape of the defect.

1. To obtain computer representations of the captured human anatomy in the form of 3-D solid or surface models, a computer-based image-reconstruction process has to be carried out on the set of 2-D tomograms. As-received CT scan data comes in DCM (Dicom) format. This data has to be first imported into MIMICS™ to form a project which is of MPJ format. This format collates the CT scan slices to form the 3-D anatomy. Each slice is 1.0mm thick which is sufficient to warrant a good accuracy for most applications. Figure 5.2 shows a sample of CT images as seen on MIMICS™.

Figure 5.2: Sample CT images of the hip region

2. Polylines are lines which represent the contour of each slice of the CT scan data. They are represented by the grey lines shown in Figure 5.3. If there is no change in the surface tomography of the defective region, the same tissue can be used to extract the surface data. Otherwise, the data obtained is either manipulated to resemble that of healthy tissue or in the case of paired organs, mirror image of the opposite tissue is used. Image segmentation using thresholding and region growing techniques are then applied to isolate data related to the region or tissue of interest and to generate the necessary 3-D surface data.

Figure 5.3: Three-dimensional surface reconstruction of the femur segment

3. The generated profiles (or contours) are reorganised axially to form a 3-D contour model. The built-in surface fitting capabilities of MIMICS™ are then utilised to regenerate the 3-D surface profile of the shaded bone segment (see Figure 5.4). Once the surfaces (inner and outer) have been generated, the data is exported in the IGES format.

Figure 5.4: Surface file of defective region

4. The surface file is first imported into Pro/ENGINEER and saved as a PRT (part) file. A surface (shown in red in Figure 5.5a) is created to cap one end of the bone segment. A merge is performed to trim the excess area which is outside the bone segment. This is repeated for the opposite end. The result is a closed volume of the surface in the shape of the patient's bone (see Figure 5.5b). It is noted that surface patching may need to be done if there are holes in the surface either due to data loss during conversion at one of the previous stages or if the patient suffers from bone loss in certain regions.

Figure 5.5: (a) Modifying the surface file (b) Closed volume of bone segment

5. Still in the Pro/ENGINEER environment, the edited surface file is appended to the pre-generated scaffold (*scaffold.prt*) which is designed to be larger than the bone segment (Figure 5.6a). A Boolean operation is performed, leaving the scaffold in the desired shape (Figure 5.6b).

Figure 5.6: (a) embedded surface model of femoral bone segment into generated scaffold structure, and (b) final shape of scaffold resembling femoral bone segment [134]

6. This final scaffold is then exported in STL format (*scaffold.stl*) for fabrication in SLS.

5.2 Fabrication of Concept Model

As a first-stage verification of CASTS, a scaffold implant of a femur segment was generated (refer to Section 5.1) and fabricated in the SLS.

Checking and repairing of STL files

The STL file of the scaffold was first checked for errors in edges or contours as well as in triangulation. This was done in Magics RPTM (Version 5.0, Materialise N.V., Leuven, Belgium). Figure 5.7 shows the pop-up window for the analysed properties of the scaffold as seen in the Magics RP environment.

Figure 5.7: Pop-up window showing the properties of the scaffold

Errors can be rectified by using the *Triangle Fixer* and the *Stitch* functions of the software. These functions allow the user to delete or repair triangles with missing vertices or edges.

The STL file was next imported into the in-built software of SLS, Sinter™ by DTM. In the case of STL files which are corrupted, Sinter™ has a function which can optimise the triangulation of STL files.

Fabrication of scaffold

The scaffold generated was the Octahedron-Tetrahedron space filling system (Configuration 1). The length of strut in each unit of the scaffold was set to 2.5mm which gives a porosity of 93% with a macro-pore size of 1.193mm (1193µm). This pore size is larger than what is required for bone cells. However, it was chosen because of the high porosity it affords.

The material used was Duraform™ Polyamide. The laser power was set to 4W. The scan speed and part bed temperature were set to default values for Duraform™, which are 200 in/s (5080 mm/s) and 165 °C respectively. The powder layer thickness of each layer was set to 0.006 in (0.152mm). Figure 5.8 shows the fabricated femur scaffold together with a RP fabricated model of the femoral head.

Figure 5.8: Fabricated models of the femoral head and scaffold structure for the femoral bone segment

Discussion

The scaffold implant fabricated has good interconnectivity and the features of the microarchitecture can be clearly seen. This is an improvement from the scaffolds created using the previous method of assembly (Section 4.6.3); in this method, the laser sinters across the boundaries of each unit cell thus maintaining a consistent quality throughout the scaffold. It is also noted that the powder inside the scaffold is easily removed using manual means. Polishing the scaffold however, is not possible because of the intricate nature of the structure.

One limitation is in the loss of shape of the implant where the unit cells are “cut off” by the femur surface as highlighted in Figure 5.9. This leaves the edges of the scaffold implant with incomplete unit cell. Also, there are bits of unattached strut pieces lying near the scaffold model. This is due to the surface irregularities of the femur shaft geometry. During fabrication, these pieces will break loose from the scaffold. Due to this defect, the

scaffold is unable to capture the full geometry of the bone. However, it can be minimised by increasing the resolution of the scaffold.

Figure 5.9: Femur implant with loose struts at the edges

Figure 5.10a shows a cylindrical scaffold with strut length l . It is seen that the large size of the unit cell causes the overall scaffold shape to be inaccurate. In Figure 5.10b, when the cell size is halved, the shape of the scaffold is seen to be more true to form. As scaffolds only require unit cells of small sizes, the loss of shape at large strut lengths need not be a consideration.

Figure 5.10: Cylinder-shaped scaffold with (a) strut length l and (b) strut length $l/2$

VI VERIFICATION OF CASTS

6.1 Fabrication Parameters

From previous work done, it is known that properties of the powder used are directly related to the parameters of the SLS system [92]. The interaction between the powder properties and system parameters determine the mechanical properties as well as the surface qualities of the part [101, 102]. Figure 6.1 illustrates how the interaction between some of the SLS parameters and the material properties influence the attributes of the fabricated model.

Figure 6.1: Relationship between process variables in SLS part fabrication

6.1.1 Machine Parameters

As can be seen from Figure 6.1, there are many parameters affecting the end-fabricated part. In this section, the machine variables affecting the fabricated part are discussed.

Andrew Number (denoted by A_N) indicates the relationship between 3 primary factors that affect the sintered part, namely: Fill laser power P , fill scan speed (also laser beam speed) V , and hatch spacing HS.

$$A_N = \frac{P}{V.HS} \quad (6.1)$$

Among these three parameters, fill laser power and fill scan speed have been identified as the two parameters that are easier to control in order to change the properties of the fabricated parts [136]. The significance of Andrew Number is in controlling the part quality as the operator switches between different systems. While the applied energy density alone is not sufficient to predict SLS process response [136], a constant Andrew number used in two different systems have been proven to obtain parts of similar qualities [92].

Fill laser power is the amount of laser power used to scan each layer of the part. It is measured in Watts (W). It is one of the factors that determines energy density ($J\ mm^{-2}$) which in turn affects the density of the part formed. With all other parameters constant, at low values of laser power, an increase in laser power usually means an increase in part density and as such, a decrease in part porosity [88, 93].

Fill scan speed is the speed of the laser beam that moves across the part bed. In SLS, it is measured in inches per second (in/s). A high scan speed might cause incomplete sintering of parts whereas a low scan speed increases processing time [88, 137].

Part bed temperature is the temperature of the powder in the part chamber. As mentioned in Section 2.4, the correct part bed temperature setting is important; a high temperature might cause the powder to melt or fuse prematurely while a low temperature can cause curling of the part. With all other factors constant, an increase in part bed temperature results in an increase in the density of the sintered part [138].

Powder layer thickness is the depth that the piston lowers for each layer and determines the thickness of each layer of the part. When the layer is too thick, the laser might be unable to sinter it completely.

Apart from the above-mentioned variables, the position of part placement on the part bed is known to affect the density of the part as well as the part accuracy [88, 138]. Orientation of the part on the other hand influences the extent of warping and amount of powder trapped within the part [139, 140]

6.1.2 Powder properties

The particle size of the powder affects surface roughness and feature accuracy. The particle size distribution influences packing and hence, the density of the sintered part. Small particle sizes produce parts with low surface roughness and good accuracy but too small a particle size can prevent the roller from spreading the powder evenly [140]. One

of the disadvantages of SLS is that complete compaction of powder is difficult to achieve. This is an advantage however when porous parts need to be fabricated [141].

The particle shape also influences the quality of the part. Powders of irregular shape sinter faster as the radii are sharper at the point of contact [88].

6.2 Generation of Scaffolds

The concept model (Section 5.2), though successfully fabricated, only made use of one possible size of a space filling system from CASTS. Further investigations need to be carried out to validate the practicability of CASTS. Figure 6.2 shows the process flow of this investigation.

Figure 6.2: Process flow of operations

The configuration tested was octahedron-tetrahedron configuration from the scaffold library. Each specimen sample fabricated was 15mm in diameter. This was intended to facilitate the cytotoxicity testing that will follow once the specimens are fabricated using biomaterials.

Using the user interface of the algorithm in Pro/ENGINEER, the configuration desired was first selected. After the selection, the layout was regenerated to activate the prompts for the secondary input. The other main inputs are the length of struts and the diameter of strut which together control the pore size and porosity. In the case of the octahedron-tetrahedron configuration, the strut length is the same throughout the unit cell and only one value needs to be specified.

For consistency of results, the diameter of the strut was fixed at 0.25mm (approximately two times the resolution of SLS) and only the strut length was varied to produce variation in the structures in terms of pore sizes and porosity. Table 6.1 is a summary of properties of the scaffolds generated.

Table 6.1: Properties of scaffolds generated

Strut length (mm)	Pore size (mm)	Porosity
1.0	0.327	0.583
1.5	0.616	0.815
2.0	0.905	0.896
2.5	1.193	0.933

The scaffold was generated to a size larger than the desired final scaffold size (see Figure 6.3a). Concurrently, a set of 16mm discs of surfaces was created in Pro/ENGINEER. The discs are varied in height from 1.0 to 2.5mm, to match one layer of unit cell for differing

strut lengths (see Figure 6.3b). Once the scaffold structure had been generated, the appropriate surface model was appended to the disc and a Boolean subtraction was performed which gave the scaffold in the shape of the disc (see Figure 6.3c & d).

Figure 6.3: Steps in Scaffold Generation (a) Rectangular block scaffold (b) Surface file of the disc (c) Scaffold with surface model embedded and (d) Final scaffold in the shape of the disc

The scaffold files (see Figure 6.4) were exported into .STL format and checked for errors in edges and contours after which, they were sent to the SLS system to be fabricated.

Figure 6.4: An example of the generated scaffolds

6.3 Duraform™ Polyamide Scaffolds

It is decided that Nylon 12, with the commercial name Duraform™ Polyamide, a standard material for SLS, should be used for the investigation. As this material has been widely studied [18, 88, 138, 142, 143] with regards to its application in SLS, this will reduce the number of variables that need to be dealt with.

The advantages of Duraform™ are uniform powder size and consistent melting point (see Table 6.2). The complete material specifications can be found in Appendix B.

Table 6.2: Material specifications – Duraform™ Polyamide

Particle shape	Irregular
Particle size range, μm	25-92
Average particle size, μm	60
Powder density, kg/m^3	590
Solid density, kg/m^3	970
Melting Point, $^{\circ}\text{C}$	186

6.3.1 Choosing Machine Parameters

As mentioned in Section 6.1, there are many parameters that are important in scaffold fabrication using SLS. However, the effects of fill laser power and fill scan speed on Duraform™ Polyamide have been intensively investigated [88, 93]. Therefore, only the other remaining parameter, part bed temperature, is taken into consideration; part bed temperature affects the degree of sintering and hence the amount of heat absorbed/conducted by the powder. Powder layer thickness is set to the maximum value as time is an important factor when many sets of experiments need to be carried out.

To investigate the effect of part placement, Phung divided the area of the part bed into a 9 by 9 matrix (Figure 6.5) [88]. He found that when the parts are placed within the 5 by 5 matrix region, the effect of the part placement on the density and part precision is minimised.

Figure 6.5: Part bed position in a 9 by 9 matrix [88]

6.3.2 Fabrication of Scaffolds

For each variation, a set of ten samples were built. Tests were carried out at part bed temperatures of 155.0°C, 157.5°C, 160.0°C, 162.5°C, 165.0°C (default value found by experiment for most Duraform™ Polyamide parts), and 175°C. The following parameters were not varied: laser power = 4W, fill scan speed = 5080 mm/s (200 in/s) (default value), powder layer thickness = 0.152 mm (0.006 in), warm up height = 6.35 mm (0.250 in) and cool-down height = 2.54 mm (0.100 in). For easy reference, the scaffold sample sets were named as shown in Table 6.3 according to the part bed temperature at which they are fabricated as well as the strut length.

Table 6.3: Naming system for specimens [144]

	1.0	1.5	2.0	2.5
Group A - 155.0°C	A1	A1.5	A2	A2.5
Group B - 157.5°C	B1	B1.5	B2	B2.5
Group C -160.0°C	C1	C1.5	C2	C2.5
Group D -162.5°C	D1	D1.5	D2	D2.5
Group E -165.0°C	E1	E1.5	E2	E2.5
Group F -175.0°C	F1	F1.5	F2	F2.5

Manual post-processing was carried out on the scaffolds to remove the loose powder. Selected specimens were also sent for ultrasonic cleaning to test if the method was more efficient. The specimens to be cleaned via ultrasonic means were selected based on the pore size and porosity of the specimens (those that matched the requirement for TE scaffolds) as well as apparent strength of the specimens.

6.3.3 Results and Discussion

All the specimens were built successfully except those in Group F. The high temperature caused the part bed to harden (see Figure 6.6) and did not yield any specimens.

Figure 6.6: Hardened part bed at 175°C [144]

It was observed that at lower part bed temperatures in the range of 155.0° C to 157.5° C, there was a higher occurrence of delamination, i.e. the bottom layer of the sample became detached. An example is seen in Figure 6.7 which shows the exposed inner struts as a result of delamination.

Figure 6.7: Sample with delaminated layer removed

The delamination was due to insufficient necking between the powder particles at the time of sintering. Delamination was also observed to occur only for samples which have 2.5 mm strut length. This is explained by the fact that as pore size and porosity of the structure increase, contact surface area between two adjacent layers decreases, making it harder for the layers to stick together.

It was also noted that at higher part bed temperatures, apparent strength of the samples increased. Studies have shown that density of the fabricated part increases with an increase in temperature [138] and this was due to higher instances of necking between the particles.

The samples obtained were cleaned following standard post-processing procedures. It was noted that for samples with low strut lengths, 1.0mm and 1.5mm, the powder trapped

inside the pores cannot be easily removed. After manual cleaning, the samples were weighed to get an overview. The weight of each sample depends on the density of the structure as well as the powder trapped within the pores. As temperature was increased, the density of the samples increased [138] and hence, so did the strength. However, the higher temperature also caused a rise in the volume of trapped powder as surrounding particles became more prone to being sintered. This observation is consistent with the results obtained from the samples as can be seen from Figures 6.8 and 6.9.

Figure 6.8: Weight distribution after manual cleaning (Strut length) [144]

Figure 6.9: Weight distribution after manual cleaning (Part bed temperature) [144]

A TE scaffold must have accessible pores while maintaining the necessary strength to act as support. In this case, the trapped powder presents a problem. The conventional way of minimising trapped powder is by changing the orientation of the part [139, 145]. As this was not feasible in this case, ultrasonic cleaning was considered as a possible solution to reduce the amount of trapped powder. Data from the graphs as well as micrographs of the samples was used to select the samples for ultrasonic cleaning. Samples with strut lengths of 2.5mm were rejected, as the pore sizes were too large. Those temperatures at too high or low were also rejected due to insufficient strength and overly clogged pores, respectively.

6.3.4 Ultrasonic Cleaning

Only Groups B1, B1.5, B2, C1, C1.5, C2, D1, D1.5, and D2 were cleaned by ultrasonic cleaning. Samples to be cleaned were placed in an empty beaker. To prevent the samples from floating up, a plastic piece was fixed to the inside of the beaker towards the bottom of the beaker. The beaker was then filled with water to cover the plastic sheet and placed inside the ultrasonic cleaning machine. The ultrasound was switched on for an hour. Once it stopped, the samples were placed in an oven at 70°C for an hour for drying. Samples were then re-weighed and the results were compared to the weight samples obtained after manual cleaning.

It was observed that the weight of the samples was only slightly reduced indicating that while ultrasonic cleaning did remove some of the trapped powder, it was not conclusive as the amount of powder removed was not very significant. Increasing the cleaning time or combining another means of cleaning with ultrasonic cleaning might increase the effectiveness.

6.3.5 Fabricated Scaffolds

Figure 6.10 shows the micrographs of a set of fabricated scaffolds. The features of the scaffolds were examined under microscope using Nikon SMZ-U Stereoscopic microscope with light unit Photomic PL 3000. Scaffolds with strut length 1.5mm and 2.0mm have reasonable pore sizes (0.616-0.905 μ m) with little trapped powder. This shows that the scaffold library and the algorithm, together with the SLS system, can produce viable scaffolds with consistent and reproducible microarchitecture.

(a) Strut length=2.5mm

(b) Strut length=2.0mm

(c) Strut length=1.5mm

(d) Strut length=1.0mm

Figure 6.10: Micrographs of fabricated scaffold samples [144]

6.4 Biomaterial Scaffolds

6.4.1 Specifications of Materials

Poly-ether-ether-ketone (PEEK) and Hydroxyapatite (HA)

The same PEEK and HA powders as well as the PEEK-HA composite blend used by Tan *et al* [76] was used for scaffold fabrication. The micrographs of as-received PEEK and HA powders were taken using Scanning Electron Microscope (SEM) to study the shape of the powders (Figure 6.11). As observed in these figures, PEEK particles are irregular in shape whereas HA particles are more or less spherical. This difference in morphology is important as it makes it possible to distinguish between the two types of materials by visual inspection.

Figure 6.11: SEM micrographs of as-received (a) PEEK and (b) HA powders [72]

Table 6.4 shows the specifications of PEEK, obtained from Victrex Plc (Lancashire, UK) under the brand name PEEK™ 150XF.

Table 6.4: Material Specifications – Poly-ether-ether-ketone

Particle shape	Irregular
Average particle size, μm	25
Powder density, kg/m^3	1320*
Glass transition temperature/ $^{\circ}\text{C}$	143
Melting temperature/ $^{\circ}\text{C}$	343

*The density of the powder is assumed to be of the same value as the solid from which it is crushed.

HA powders used in the research meet the requirements of the ASTM F 1185-88 and have a particle size distribution of at least 90 wt% of particles below the size of $60\mu\text{m}$ as determined by Coulter Counter analysis. Table 6.5 shows the material specifications for HA.

Table 6.5: Material Specifications – Hydroxyapatite

Particle shape	Spherical
Particle size range, μm	5-60
Powder density, kg/m^3	3050

Polycaprolactone (PCL)

PCL used in this project was purchased from Solvay Interlox Pte Ltd, UK under the brand name CAPA[®] 6501. The powder has a molecular weight of 50000. Scanning Electron Micrography shows the particles to be irregular in shape (Figure 6.12).

Figure 6.12: SEM Micrographs of as-received PCL [146]

Table 6.6 summarises the material specifications of PCL.

Table 6.6: Material Specifications – Poly-ε-caprolactone [146]

Particle shape	Irregular
Particle size distribution: <100 µm	99%
Powder density, kg/m ³	1.15
Glass transition temperature/ °C	-60
Melting temperature/ °C	60

6.3.2 Pure PEEK scaffolds

Previous PEEK and PEEK-HA composite samples built in the SLS are only in the form of solid discs. Therefore, it is necessary to ascertain the feasibility of incorporating these materials into CASTS. As a start, pure PEEK scaffolds were fabricated before experimenting with the composite material.

The scaffolds built were based on the same STL files used for Duraform™ samples (see Section 6.1). As seen with Duraform™ samples (see Section 6.2), scaffolds with strut length 2.0mm had very large pores (>1000µm) which are not suitable for cell seeding.

The large pores may cause the cells to slip through and settle at the bottom instead of attaching to the scaffold surfaces. Also, in the event that some cells do adhere to the surfaces and proliferate, this will result in a weak cell mass as cells only tend to grow along surfaces which is not desirable. Therefore, for this stage, only three varying strut lengths were considered: 1.0 mm, 1.5 mm, and 2.0 mm.

For this investigation, a laser power of 17W was used at part bed temperature 140°C and scan speed 5080 mm/s (200 in/s) [76]. The reason for using one of the lower laser powers is to ensure that the energy density (i.e. temperature) incident on the part during sintering does not become too high such that the properties of the material become altered.

The first batch of specimens was built at a layer thickness of 0.152 mm (0.006 in). The parts built were found to be fragile and hard to handle. This could be due to the inability of the laser power to sinter each layer completely at such thickness. Therefore, a second batch was fabricated with the layer thickness value reduced to 0.10mm (0.004 in).

Results and discussion

In general, the fabricated scaffolds exhibit well-defined microarchitecture, indicating the possibility of incorporating biomaterials into CASTS, even though the structural integrity of the PEEK scaffolds was observed to be not as good as those fabricated with Duraform™. This is expected since Duraform™ is a commercial material with optimised settings in SLS while PEEK is a new material. The advantage of PEEK scaffolds over Duraform™ scaffolds is in the reduction of powder trapped within the pores and also the ease of powder removal. This may be attributed to the small particle size of PEEK powders, which at 25µm, is less than half the average size of the Duraform™ powders.

Scaffolds with strut length 1.0 had the least visible pores. This was due to 2 reasons; the theoretical pore sizes are in the order of 300 μ m and also, as the pore size gets smaller, the powder is harder to remove (see Figure 6.13).

Figure 6.13: (a) Top and (b) bottom views of a scaffold specimen with strut length 1.0mm

Curling of the edges was also observed in this set of specimens. This phenomenon usually occurs when there is a substantial temperature difference between the sintered part and the powders in the part bed surrounding the part. In this case, the small features require the laser to sinter repeatedly within a small region. The increase in thermal conduction in the region raises the temperature of that region significantly higher than the surrounding part bed powder. The part also possibly retained the heat from the laser hence creating a temperature gradient. Large thermal gradients result in curling of the edges [75]. The curling in turn induced the roller to drag the previously fabricated layers, causing disparities in alignment in the subsequent layers (Figure 6.14), and thus compromising the dimensional accuracy of the part.

Figure 6.14: (a) Misaligned layers (b) Close-up view

Scaffolds with strut length 1.5mm showed most favourable results with intact struts and well-defined pores (Figure 6.15). The intended architecture was clearly visible under the microscope. This is further proven in Figure 6.16 where the top few layers have been removed to expose the interconnected struts within the specimen.

Figure 6.15: Scaffolds with strut length 1.5mm (a) Top view (b) Bottom view

Figure 6.16: Sample with top layers exposed to show the inner structure

Broken inner struts were observed in scaffolds with strut length 2.0mm (Figure 6.16). This could be due to insufficient strength holding the struts together as there are now fewer struts in a given area.

Figure 6.17: A scaffold with strut length 2.0mm (a) Top view (b) Bottom View

Delamination was observed for the first layer (0.10mm) for scaffolds with strut length 1.5mm and 2.0mm. This was more pronounced in scaffolds with strut length 2.0 mm as the first layer was completely separated from the rest of the scaffold even before the part was removed from the part bed. For scaffolds with strut length 1.5mm, the first layer only

came off during handling. This indicated that the bond between the first layer and the next is weak. However, the delamination of the first layer did not affect the overall structure of the scaffold as seen in photos taken under the microscope. Figure 6.18 shows the delaminated first layer of a scaffold.

Figure 6.18: Delaminated first layer

6.3.3 PEEK-HA Composite Scaffolds

After successfully sintering the PEEK scaffolds, PEEK-HA composite scaffolds were fabricated. The composite blend is made up of 90% weight PEEK with 10% weight HA. The physical blend was produced by using PEEK as a base material and adding in HA gradually in the roller mixer [76]. Figure 6.19a shows the distribution of HA particles (highlighted in red) in the composite after blending.

Figure 6.18: SEM micrographs of PEEK-10% wt HA (a) before and (b) after sintering [76]

After processing in SLS, it was seen that the HA particles were trapped in the interconnected PEEK matrix (see Figure 6.18b). This is only possible because PEEK has a much lower melting point compared to HA and the temperatures that the composite is exposed to in the SLS are not high enough to affect the HA particles.

Experiments showed that scaffolds with strut length 1.5mm were most promising in terms of structural shape as well as theoretical pore size ($\sim 600\mu\text{m}$) and porosity ($> 80\%$). Therefore, a set of scaffolds with strut length 1.5mm was fabricated using the composite. The same parameters used for sintering PEEK were used to process the PEEK-10% wt HA composite i.e. laser power of 17W at part bed temperature 140°C , scan speed 200 in/s (5080 mm/s), and layer thickness of 0.004 in ($\sim 0.10\text{mm}$).

Results and discussion

The scaffolds showed similar structure integrity and pore sizes as the pure PEEK scaffolds. The pores were also clearly visible under microscope (see Figure 6.19).

Figure 6.19: Top and bottom views of the PEEK-HA composite scaffold

One observation made was the “balling effect” present in the fabricated specimens. This was not observed in the Duraform™ scaffolds or the pure PEEK samples. Nelson [92] attributed this to surface tension present in the powder bed. As PEEK particles act as the binder and the HA particles remain in the solid phase, droplets of binder form on the scanned surface. Figure 6.20 shows the inner struts which have been removed from the scaffold. The balling effect was visible in some of the clusters of struts (highlighted in circles).

Figure 6.20: Balling effect seen in the inner struts

6.3.3 Polycaprolactone (PCL) scaffolds

Polycaprolactone, being a biodegradable material, has a natural advantage over PEEK. As PCL is a relatively new material with regards to SLS, a scaffold model was first fabricated to ascertain the feasibility of fabrication. Figure 6.21 shows the CAD model of a truncated octahedron space filling system. The scaffold measures 35mm by 35mm by 35mm and with a strut radius of 0.28mm. The number of cells in each direction is 4.

Figure 6.21: CAD model of truncated octahedron space filling system

The scaffold model was fabricated using the following set of parameters: laser power = 3W, fill scan speed = 5080 mm/s (200 in/s) (default), powder layer thickness = 0.102 mm (0.004 in), warm up height = 6.35mm (0.250in), and cool-down height = 2.54mm (0.100 in). PCL disc scaffolds were fabricated using the same set of parameters. Similar to the biocomposite scaffolds, the strut length was 1.5mm with strut diameter 0.25mm.

Results and Discussion

A sample of fabricated scaffold model is shown in Figure 6.22(a). The models were found to be spongy in nature and structurally uniform. However, in one of the models, there was

a slight shift in the first layer when the roller of the SLS dragged the cross section. This resulted in a defect in the model (Figure 6.22(b)).

Figure 6.22: (a) Fabricated poly- ϵ -caprolactone scaffold model (b) Defect

A sample of the fabricated PCL scaffold is shown in Figure 6.23. In general, it was found that PCL scaffolds exhibited better structural integrity and higher strengths than PEEK or biocomposite scaffolds. There was also a lower percentage of delamination. When observed under the microscope, there was evidence of crystallinity in the scaffolds. However, due to the much larger particle size of PCL, the struts were found to be much thicker than those of PEEK scaffolds.

Figure 6.23: Poly- ϵ -caprolactone scaffold

In Chapters 7 and 8, mechanical characterisation and in vitro studies using PCL scaffolds are discussed. PCL scaffolds are used for this purpose as they have better apparent strength than PEEK or PEEK-HA composite scaffolds and they have also been used to grow bone.

VII MECHANICAL CHARACTERISATION

As discussed in Chapter 2, scaffolds must have mechanical properties compatible to that of native tissue. This is especially important in load bearing tissues such as bone. Hence, mechanical properties of sintered polycaprolactone were determined. These values were then used to simulate the behaviour of unit cells of three different configurations in the parametric library. As expected, the mechanical properties varied with polyhedral shape. Finite element analysis was also applied to investigate the behaviour of the different configurations under loading. Lastly, an experiment was set up to explore the effect of build orientation on the mechanical properties of the scaffolds. Findings confirm that not only can the mechanical properties of scaffolds vary with change in unit shape, the build orientation also plays a part.

7.1 Mechanical properties of sintered polycaprolactone

There has been little research carried out to measure the mechanical strength sintered PCL. To investigate the behaviour of sintered PCL, rectangular blocks of PCL measuring 12.7 mm in diameter and 25.4mm in length were fabricated according to ASTM standard D695 [147]. Following the ASTM standards, 5 samples were tested using Instron Model 5569. A 50kN load cell was used at a compression rate of 1mm/min. The test was run up to a strain rate of 40%. Figure 7.1 shows the stress-strain curve of PCL up to 40% strain rate.

Figure 7.1: Stress-strain curve for 5 sample of sintered PCL fabricated based on ASTM D695

From the graphs, the results were consistent for all samples. The initial slow rates of increase in stress maybe an indication of the microporous nature of the specimen. Between the strain percentages of 15 and 20%, the gradient of the curve increased steadily which is another indication of cellular plastic behaviour. However, as the behaviour of the stress-strain curve is more similar to rigid solids, the gradient of the curve between 5 and 15% of strain was used to determine the elastic modulus of the curve.

In order to obtain the Poisson Ratio of the material, dog bone samples were built according to ASTM standard D638. However, due to the nature of the material, there was sliding between the different layers, causing the layers to mis-align (Figure 7.2).

Figure 7.2: Tensile sample of PCL

Hence, Poisson ratio had to be estimated. Typically, semi-crystalline polymers have a Poisson ratio of about 0.41-0.45 [148]. PCL is estimated to have a Poisson ratio of about 0.42. Hence, shear modulus, G can be calculated as:

$$G = \frac{E}{2(1 + \nu)} \quad (7.1)$$

where E is the elastic modulus obtained from the stress-strain curve and ν is the Poisson ratio of the material

Octahedron-Tetrahedron Scaffolds

To carry out mechanical characterisation of scaffolds, one of the configurations, octahedron-tetrahedron was generated using CASTS. The strut length and diameter were set at 1.25 mm and 0.25mm respectively. The scaffold generated was trimmed to 12.7 mm x 12.7 mm x 25.4mm using Boolean operations and exported in the STL format. The build parameters were the same as those used for the solid samples.

Five scaffold samples were tested for a strain rate of 40% at a compression rate of 1mm/min. The graphs (Figure 7.3) show that the scaffold had much lower compression

strengths than that of solid samples. This is expected as the scaffolds, being cellular solids, are much more porous.

Figure 7.3: Stress-strain curves of 5 samples of Octahedron-tetrahedron scaffolds with $l=1.25\text{mm}$ built according to ASTM D695

7.2 Finite Element Analysis

Finite element analysis is used to study the behaviour of the different polyhedral shapes under loading. Three configurations were chosen, namely, octahedron-tetrahedron configuration, cuboctahedron-octahedron configuration, and the square prism configuration. The investigation was carried out in two stages. Firstly, individual cells were modelled as solids and a unit load was applied to observe the stress distribution within each unit cell. In the second stage, the behaviour of the assembled scaffolds under loading was simulated. For both sets of investigation, Abaqus version 6.4.1 was used.

In order to estimate the correct Poisson ratio, ν , of sintered polycaprolactone, a finite element analysis was done on a solid model (12.5x12.5x25mm) assumed to be isotropic with the following properties: $E = 10.50 \times 10^6$ and $\rho = 1.153 \text{ kg/m}^3$. Modulus of the material was determined from the compression results carried out and shear modulus was calculated using Equation 7.1. Density of the material was measured using an air pycnometer [146].

The solid was meshed with C3D8R, fully integrated linear hexagonal elements. There were a total of 396 nodes and 250 elements. A load was applied to the top surface such that the top moves with a downward velocity of 1mm/min while the bottom surface was constrained with zero degrees of freedom. Loading was uni-directional and uniform across the whole surface. Figure 7.4 shows the solid under constraints and loading (a) and after meshing (b).

Figure 7.4: (a) Solid part under loading and constraints (b) After meshing

Poisson ratio, ν , was varied between 0.41 to 0.45 and corresponding stress-strain curves were plotted and compared with the results of compression testing. It was found that a Poisson ratio of 0.42 produced a stress-strain curve that is almost identical to that of the compression test. This Poisson ratio gives a shear modulus, G , of 3.697×10^6 . Figure 7.5 shows the simulated stress-strain graph.

Figure 7.5: FEA simulation of solid PCL under loading

7.2.1 Stress distribution in unit cells

To study the stress distribution in unit cells, the cells were generated using the CASTS scaffold library and algorithm. They were then exported as solid IGES files to Abaqus. The material was assumed to be isotropic and assigned the following properties (from Section 7.1): $E = 10.50 \times 10^6$, $\nu = 0.42$, $G = 3.697 \times 10^6$ and $\rho = 1.153 \text{ kg/m}^3$.

All the three different unit cells were meshed with C3D4, four noded tetrahedral elements. The mesh resulted in 2120 nodes and 3387 elements in the octahedron-tetrahedron unit cell, 1658 nodes and 2746 elements in the cuboctahedron-octahedron unit cell, and 125 nodes and 1492 elements in the square prism unit cell. Similar to the solid model, a uni-directional compression load was applied to the top of the unit cell while the bottom was constrained with zero degrees of freedom.

Figure 7.6 shows the Von Mises stress distribution for (a) octahedron-tetrahedron unit cell, (b) cuboctahedron-octahedron unit cell, and (c) the square prism unit cell. The highest stress levels were reached by the octahedron-tetrahedron unit cell. However, a large fraction of the volume has stress levels of below 2.69Pa under unit loading.

Figure 7.6: Stress distribution of different polyhedral cells under unit loading

In square prism unit cells, the stress was observed to be evenly distributed with the exception of a few elements in the vertical struts just below the surface. These are likely

to be points of inflexion should buckling occur. In vivo, the stress experienced by cells due to gravity is as low as 0.05Pa (0.5 dyn/cm²) [35] which means the forces applied are much lower than that is used in this simulation. Even though shear forces exerted by blood flow are much larger, scaffolds are unlikely to experience such high forces; as such devices are not likely to be in direct contact with the blood stream.

7.2.2 Behaviour of scaffolds under loading

To obtain stress-strain curves of scaffolds, unit cells of height 1.25mm are generated using CASTS and exported as IGES wireframe. After importing into Abaqus, the units are repeated to model scaffolds of 125 cells, based on the work of Meguid *et al* who used finite element analysis to study deformation of metallic foams [149]. Wireframe models have to be used as shells and solids require a high number of elements and nodes and are not feasible for simulation of scaffold assemblies. Figure 7.7 shows a unit cell of square prism configuration (left) and the assembled scaffold of 125 unit cells (right).

Figure 7.7: Square prism configuration in wireframe: Unit cell (left) and Assembled scaffold (right)

PCL was again assumed to be isotropic with the following properties: $E = 10.50 \times 10^6$, $\nu = 0.42$, $G = 3.697 \times 10^6$, and $\rho = 1.153 \text{ kg/m}^3$. The bottom surface was constrained from any

movement and a velocity of 1mm/min was applied to the top surface of the scaffold to mimic the compression test. The simulations were carried out for each of the three configurations for a strain rate of 10%. B31, three-dimensional 2-noded linear beam elements, were used for all three configurations. Each beam element was assigned a circular profile with a diameter of 0.25mm. A maximum number of 1000 increments were allowed to obtain more accurate results.

Meshing produced a total of 1658 nodes and 2746 elements in the cuboctahedron-octahedron configuration, and 125 nodes and 1492 elements in the square prism configuration. Upon completion of simulation, the data was exported and the stress-strain curves were compared for the different scaffold configurations (Figure 7.8). The graphs show that the scaffolds generated from different unit cell types behave differently under loading.

Comparing the simulation with the compression testing of octahedron-tetrahedron scaffolds, it was observed that the square prism scaffolds displayed a slightly lower compressive modulus than the octahedron-tetrahedron scaffolds while the cuboctahedron-octahedron scaffolds have a much lower modulus. This is in accordance with the stress distribution patterns displayed under unit loading.

Figure 7.8: Simulated stress-strain curves for cuboctahedron-octahedron and square prism scaffolds

7.3 Effect of Build Orientation

As discussed in Section 7.2, the main advantage of using polyhedral shapes for scaffold design is that a large number of configurations to vary the mechanical properties of the scaffold can be obtained by varying parameters such as the type of unit cell, the biomaterial used, and the RP processing technique used. Even within a fixed set of the above-mentioned parameters, the mechanical properties may be varied by factors such as build orientation.

For SinterstationTM 2500, the x-y resolution is 0.127mm while the minimal layer thickness is 0.10mm hence the resolution of small features may also be affected by build orientation. To investigate the effect of build orientation on scaffolds, rectangular blocks of octahedron-tetrahedron scaffolds were generated and fabricated in PCL using SLS. The strut length was set at 1.25mm and the strut diameter at 0.25mm. Scaffolds were built in three different orientations (Figure 7.9).

Figure 7.9: The different build orientations as defined by the plane in which the shaded surface lays. From left: X-Y plane, Y-Z plane and X-Z plane

7.3.1 Imaging techniques

For analysis through imaging techniques, cubes of 12.0mm x 12.0mm x 12.0mm were fabricated. Figure 7.10 shows the fabricated cubes. Surface feature details were seen to be different from each other. The struts of the scaffolds built in XY plane were observed to be larger than those of scaffolds built in the other two orientations.

Figure 7.10: Scaffold cubes built in different orientations

One challenge with scaffolds fabricated by SLS is the removal of powder as mentioned in Section 6.3. With PCL scaffolds, powder removal was relatively easy compared to materials such as DuraformTM. As the PCL parts fabricated were elastic, powder trapped within the scaffolds were easily removed by using a sieve shaker.

To check for broken struts and residual trapped powder within the scaffolds, microcomputed tomography (micro CT) was carried out using SkyScan-1074 Portable x-ray micro-CT scanner. Samples were first scanned in their entirety (slice thickness of 16 μ m) and then zoomed in to the view the volume at the centre (slice thickness of 8 μ m). Results showed that even in XY-plane samples with its larger sized struts, there was a through network of pores. Figure 7.11 shows a scanned slice of the whole cross section of

the scaffold built in XZ-plane and on the right, a portion of the reconstructed three dimensional scaffold.

Figure 7.11: A scanned micro-CT section of the scaffold oriented in XZ plane

7.3.2 Compression testing

Samples for compressing testing of scaffolds of octahedron-tetrahedron configuration were designed to be rectangular blocks of 12.7mm x 12.7mm x 25.4mm. The samples were tested similarly (as solid samples) using a strain rate of 1mm/min. The results of the compression tests are shown in Figures 7.12.

Figure 7.12: Stress-strain curves of octahedron-tetrahedron scaffolds built in different orientations

Results obtained for each orientation were consistent with characterisation samples in Section 7.1. The stress-strain curves of scaffolds fabricated in orientation planes of YZ and XZ displayed a higher likeness to the behaviour of cellular materials. This can be explained by that fact that for these samples, resolution of the features were higher and hence, the struts were smaller and more prone to buckling. Also, in these samples, force applied is parallel to the plane of sintering. This causes fractures to form in between the layers and weaken the structure.

It was observed that while the stress-strain curves of samples built in YZ and XZ planes were similar, YZ samples were found to reach the plastic region of the curve at a lower strain rate. When samples were compared after compression testing, YZ samples were found to have obvious fracture lines along the longitudinal axis, highlighted by the arrow

in Figure 7.13. XY samples were also found to have collapsed struts around the middle of the scaffold (circle) but XZ samples had no visible damage and recovered most of its elasticity after the compression test. Solid samples also underwent plastic deformation as indicated by the decrease in height and increase in width of the base.

Figure 7.13: Samples after compression testing (a) Solid (b) Scaffold in XY plane orientation (c) Scaffold in YZ plane orientation (d) Scaffold in XZ plane orientation

Figure 7.14 gives a comparison between the compressive strengths of the scaffolds built in different orientations with respect to the solid PCL part.

Figure 7.14: Histogram comparing the maximum compressive strength

VIII IN VITRO STUDIES

Preliminary in vitro studies were carried out to investigate the response of living cells to sintered PCL scaffolds and to investigate the influence the different shapes of polyhedra have on cytotoxicity and cell proliferation. A cell line, Saos-2, was identified for *in vitro* studies. The cell line was purchased and passaged, and standard tests for bacteria, fungi and mycoplasma were carried out periodically. A seeding density test was conducted over a period of 7 days to gauge the optimal seeding density of the scaffolds. Three different configurations of scaffolds were then seeded with cells and observed for a duration of 6 weeks. Test results indicated that pore shapes do influence the rate of proliferation of Saos-2 cells. All experiments were conducted in DNA Centre, National Institute of Education, Singapore.

8.1 Saos-2 Osteosarcoma cells

A transformed cell line is used in this study as it has higher growth rates and higher cell densities. Transformed cell lines are also easier to maintain in terms of media and serum requirements. Primary cultures, on the other hand, are difficult to acquire and maintain and are restricted to a finite number of passages [150]. Saos-2 cells are originally harvested from an osteosarcoma of an 11-year old Caucasian female patient in 1975. They can be propagated using currently existing culture methods. Cells used in this study are purchased and shipped from ECACC (Catalogue No. 89050205).

The morphology of Saos-2 is epithelial-like. Saos-2 cells display osteoblast-like characteristics and produce bone morphogenetic proteins [151]. Unlike most other Osteosarcoma however, Saos-2 cells are also osteoinductive; they have been found to

release bone-inducing agents into the media in in-vitro cultures [151, 152]. In vivo, 10×10^6 saos-2 cells are enough for 100% bone induction in nude mice [151].

Surface characteristics of implants have been shown to have an effect on cell proliferation and differentiation of Saos-2 cells. While there was no significant difference in rates of cell apoptosis between rough or smooth surfaces, it was found that these cells proliferate better on smooth surfaces while surface roughness promotes differentiation towards an osteoblastic phenotype which will enhance bone healing and eventual osteointegration with the surrounding tissue [153].

The growth cycle typically follows three phases: (a) the lag phase, (b) the log phase, and (c) the plateau or the stationary phase (Figure 8.1) [154]. In cell cultures, there exists another phase called the death phase if there are not enough nutrients provided for the cells that have reached the stationary phase.

Figure 8.1: A typical sigmoid growth curve showing (a) the lag phase (b) the log phase and (c) the stationary phase

The lag phase

When the cells are first seeded onto the substrate, they will have to attach themselves to the substrate and spread out. Enzymes and proteins accumulate in preparation for cell division. There is little or no growth in terms of cell numbers [155].

The log phase

During this period, there is exponential increase in cell number. The largest phase of the growth cycle, this phase starts after the lag phase and continues till the cells reach confluence. During this phase, the cells are at steady state; they are at their most uniform and have a high viability. The growth fraction of the cells is very high (80-90%) [155]. The actual length of this time is dependent on a few factors such as seeding density, the growth rate of the cells and the density at which cell proliferation is uninhibited.

The stationary phase

This phase occurs after the log phase. During this stage, the culture becomes confluent i.e. all of the available area of the substrate is occupied by the cells and the cells are in contact with each other. The growth rate decreases and the growth fraction of the cells is much lower than that in the log phase (0-10%). The cells become less motile and may become aligned in orientation. Growth may stop after one or two more doublings. This is termed density-dependent inhibition [155].

It is noted that transformed cell lines such as Saos-2 may not be sensitive to density-dependent inhibition and this may cause them to continue to proliferate even after they have become confluent [156] so long as there are enough nutrients. This results in higher cell densities at the plateau phase [155].

8.2 Cell Culture and Maintenance

Figure 8.2 shows the process flow in cell culture and maintenance used in this study.

Figure 8.2: Process flow in cell culture and maintenance

8.2.1 Basic reagents and equipment

Foetal Bovine Serum (FBS) (Gibco® Catalogue No. 26140087) was added to Alpha-Minimal Essential Media (α MEM) (Gibco® Catalogue No. 12561056) to make up 10% of the complete media. In addition, 100 U/ml of Penicillin and 100 μ g/ml of streptomycin (Gibco® Catalogue No. 15140148) were added as anti-biotics. Phosphate Buffer Saline used has a pH value of 7.4 (Gibco® Catalogue No. 10010023). All cell culture work was carried out in Gelman Class II Biosafety cabinet. The incubator used was JISICO J-2000 set at 36.5° Celsius and 4.0% CO₂. Safety regulations with regards to animal cell culture [155] were observed at all times.

Foetal Bovine Serum (FBS)

Some serum (5-20%) is necessary for optimal cell growth. Serum contains a complex mixture of biomolecules which supports the survival and proliferation of cells. Its components include plasma proteins, peptides, lipids, carbohydrates, and minerals as well as several growth factors which regulate proliferation and differentiation of cells in culture. These components provide nutrients, stimulate cell growth, promote attachment and spreading onto substrate, inhibit proteases, and also protect cells against mechanical damage. Serum itself also acts as a pH buffer [150].

Media

Media is the main source of nutrients for cultured cells for sustained continuous cell growth [155]. α -MEM is modified from Eagle's minimal essential medium (MEM) and is commonly used for permanent cell lines with the addition of some serum [150]. Developed in 1971, α -MEM contains extra components such as ascorbic acid and L-

glutamine. Ascorbic acid is believed to be necessary for mineral formation of Saos-2 cells [108]. A pH indicator such as Phenol Red is usually added to media for easy indication of pH [155].

Anti-biotics

Anti-biotics are necessary in cell cultures as a precaution against infection as sterilisation or equipment may not be enough to get rid of all bacteria. Infected cultures display a rapid drop in pH noticeable through colour change of media from orange to yellow. Penicillin-streptomycin is used against gram-positive and gram-negative bacteria. The concentration used in this study follows that of work done by Ciapetti *et al* [108].

Phosphate saline buffer (PBS)

Optimal pH for cell growth differs among the strain of cells with transformed cell lines having a range of pH 7.0 to 7.4. Buffering for media is required in transformed cell lines as at high cell concentrations, the amount of CO₂ released by the cells could cause the pH to drop [155].

Incubation Temperature

The temperature of incubation for cultures depends on the organism from which it is derived as well as the organ. For mammalian cultures, the temperature is typically set slightly below the body temperature for safety reasons.

8.2.2 Resuscitation of frozen cells

Upon receipt of shipment, the frozen ampoule of osteosarcoma cells was allowed to thaw at room temperature. The ampoule was agitated until all the contents had thawed. Complete media was warmed to 37°C using a water bath. The supernatant was carefully removed using a micropipette to get rid of the freezing agent while taking care not to disturb the cells. 1 mL of complete media was added to the ampoule and the cells were resuspended. Meanwhile, a 25cm² flask was filled with 4mL of complete media. After resuspension, the entire contents of the ampoule were transferred to the prepared flask and the flask was incubated.

The flask was checked for cell adhesion and death rate after one day and media was changed if necessary.

8.2.3 Passaging

Saos-2 cells were found to have a high growth rate and reach confluence in about 3 days. Cells were examined under an inverted microscope (Olympus CKX41 fitted with C5060 wide zoom camera) and subcultured at 70-90% confluence.

During subculture, cells were first washed with 10% PBS solution after the old media was removed. The media was then replaced and a cell scraper was used to detach the cells from the flask. Cells were resuspended to break up the dense clusters and then split 1:3 into new flasks. Each cell culture flask was used no more than 3 times to maintain consistency.

8.2.4 Cell counting

A Haemocytometer, shown in Figure 8.3, was used to count the cells grown in monolayers. A haemocytometer consists of a thick base plate made of special optic glass on with four longitudinal grooves along the central third of its top surface and a cover slip. The surfaces of the supports are highly polished for better reflectivity. The central support or the counting chamber is 0.1 mm deep and the counting grids are edged onto it. When the cover slip is placed on top of the supports and liquid is pipetted into the grooves, a capillary pull draws the solution into the counting chamber.

Figure 8.3: Haemocytometer

Prior to counting cells, both the haemocytometer and the cover slip were cleaned with ethanol and dried. After drying, the cover slip was placed on top of the counting chamber of the haemocytometer.

Cells were washed first with 10% PBS solution and fresh media is added to the culture flask. Cell scrapers were used to detach the cells from the flask and resuspended. 50 μ L of the suspension was transferred into a tube containing 50 μ L of NaCl solution and agitated to ensure uniform mixing. This dilution step was to ensure that cells did not overlap each other in the counting grid. 50 μ L of this solution was then transferred to 50 μ L of trypan blue. Trypan blue was used for vital staining to ensure that only live cells were counted; the stain was taken up by the nuclei of dead cells. The diluted mixture was then micropipetted into one of the grooves of the haemocytometer until the counting chamber has been completely filled. Care was taken to ensure that there was no overfilling or spillage. The haemocytometer was placed under a microscope and cells in 5 of the 1mm² areas were counted and added up (Figure 8.4).

Figure 8.4: Counting grid with labelled areas for counting

Cell density was calculated using the formula below:

$$\text{No. of cells per mL} = \frac{\sum N}{5} \times \text{Dilution Factor} \times 10^4 \quad (8.1)$$

where N is the number of cells counted per square and dilution factor is 4.

To minimise statistical error, each sample was counted three times and the averaged value was taken as the final count. Before counting, it was ensured that the cells were not allowed to settle and the suspension was well mixed prior to sampling. Also, if it was observed that more than 10% of the cells in the counting chamber were still in clumps and were not well separated, a new sample was taken from the cell suspension. Should cells fall on the borders of the grids, only those on the top or left lines were counted so as to eliminate duplicate counts.

8.2.5 Testing for contamination

Cultures that have been contaminated may exhibit different morphology or take a longer time to reach confluence. Even if these characteristics remain constant, contaminated cells may not perform physiological functions in a normal manner. Hence, it is crucial that cultures are tested against contaminations at regular intervals.

Bacteria and Fungi

Contamination by bacteria or fungal spores is easily detected by an increase in turbidity or change in colour of the media due to a drop in pH. In addition, many types of fungi infections are visible either through the naked eye or under light microscope. Fungi are usually large and appear budded. While the cells may survive for a while, they will eventually die [157].

Stain kits are also available for contamination testing. In this study, cultures were checked every 3 days for microbial contamination under microscope and also routinely tested

using the stain kit. Suspected cultures were isolated and observed for a week and discarded if found to be infected.

The stain kit used in this study was acquired from Molecular Probes (Catalogue No. C-7028) and the experimental protocol was followed as per specifications. To test for fungi contamination, a 5mL sample of media was taken from the culture and transferred to a centrifuge tube. The tube was centrifuged at 1000xg for 15 minutes. Meanwhile, 250mg of bovine serum albumin (BSA) and 0.88g of NaCl (saline) were dissolved in 100mL of distilled water. The resulting BSA-saline solution was filter-sterilised to prevent contamination and to remove particulates.

The tube was removed from the centrifuge and the supernatant was discarded. This served to remove nucleic acids and media components which may cause high level background fluorescence. The pellet was resuspended in 200 μ L of BSA-saline solution. A 20 μ L of the sample was applied onto a slide which has been cleaned with ethanol. The slide was allowed to dry at 37°C for 10 minutes. Next, the sample was heat fixed by passing over a weak open flame with the sample side up while avoiding overheating the slide. This was repeated three times.

Detection of bacteria

50 μ L of BSA-saline solution was pipetted onto the fixed cells ensuring that the whole sample was covered. The slide was left to stand for 5 minutes and the solution was removed. Lyophilised wheat germ agglutinin, Texas Red[®]-X red (WGA-TRX) fluorescent gram positive stain was reconstituted in 1mL of 0.1 M sodium bicarbonate (pH 8.3) buffer. 5 μ L of the component was then diluted with 475 μ L of BSA-saline. 50 μ L

of this diluted solution was added to the slide and left to stand for another 5 minutes before the solution was again removed.

SYTO[®] 9 green fluorescent stain was diluted to 5:95 ratio in filtered distilled water. 10 μ L of this solution was added to the slide. The slide was then washed with 50 μ L of BSA-saline to minimise background fluorescence. A coverslip was mounted onto the slide and sealed. The slides were first viewed at an excitation wavelength of 494nm [158] to check for both gram-positive and gram-negative bacteria; any bacteria present will be stained bright green fluorescent. Dust or cell debris may also give out green fluorescence but they are typically irregular in shape.

The slides were then viewed using a Texas Red filter set (595nm) [158] to check for gram-positive bacteria which will be stained bright red fluorescence. Fungi may also give out fluorescence under these stains but they can be easily distinguished by the size and appearance.

Detection of fungi

Calcofluor[™] White M2R is diluted in 100 μ L of water. This component is taken up by fungal cell walls and gives out blue fluorescence. 10 μ L of the diluted stain is pipetted onto the fixed cells. A cover slip was applied and the slide is viewed under fluorescent microscope set at a filter set between 352 to 358 nm [158]. Under these conditions, yeast and other fungi appear bulbous and filamentous fungi are also stained bright blue fluorescent. Bacteria is usually unaffected by this stain but if stained, will be less bright and much smaller in size in comparison to fungi.

Mycoplasma

Mycoplasma are a group of small, free-living prokaryotes which lack a cell wall [157, 159]. Mycoplasma infection has long term effects including changes in morphology, diminished growth rate and altered metabolism of the cells in culture [157]. Unlike bacterial or fungi infection, infection of cultures by mycoplasma is difficult to detect: while some strains of mycoplasma may cause apparent changes in terms of morphology or growth rate, other strains may actively proliferate and metabolise in cultures without detection. Thus, specialised techniques are required for routine checking of cultures for infection [160].

Advances in life science have made available detection kits for mycoplasma. The kit used in this study was MycoFluor™ Mycoplasma Detection Kit (Invitrogen Catalogue No. M-7006). This method makes use of a fluorescent nucleic acid stain. To test for cell culture, cells were first seeded onto microscope slides and covered with media. These slides were placed in petri dishes and incubated till a medium density (less than 75% confluence) has been reached.

The concentrated MycoFluor™ reagent was warmed to room temperature and vortexed to remove precipitates. The reagent was then diluted 1:19 parts to culture media. The diluted reagent was added to the slides and incubated for 30 minutes at room temperature. The slides were aspirated and washed 3 times with distilled water, allowing to dry completely after the final wash. Coverslips were placed onto the slides with the use of mounting medium.

The slides were viewed under an fluorescence filter. Control slides provided in the kit were first examined for comparison before the actual slides were viewed. In infected cultures, both the nuclei and cytoplasm were stained (Figure 8.5(b)) while in healthy cultures, only the nuclei was stained (Figure 8.5(d)). Figure 8.5(a) and (c) show the cells under bright field.

(a)

(b)

(c)

(d)

Figure 8.5: (a)(b) Positive control and (c)(d) Healthy sample

8.2.6 Freezing

Cells are routinely frozen for banking purposes so that in case of contamination, there is ready stock available for continuation of cell cultures. When cells are frozen, the resulting ice crystals and osmotic pressure can cause cellular damage. Hence, the use of cryoprotectants such as Dimethylsulfoxide (DMSO) is necessary [160].

Before cell banking, cultures were grown in 75cm² flasks in monolayers till confluence has been reached. On the day of banking, media in the flask was replaced with fresh media. 15% DMSO was prepared fresh with 85% of complete media and set aside. Cells were detached using cell scrapers and transferred to 15mL centrifuge tubes. The tubes were centrifuged at 2000g for 10 minutes. The supernatant was removed using a pipette and the cells were resuspended in 3mL of fresh media.

0.5mL of the cell suspension was pipetted into each of the cryotubes and 0.5mL of 15% DMSO was added to each tube. The cryotubes were labelled and put into Styrofoam boxes to achieve slow freezing. The boxes were placed overnight in a 80°C freezer before being transferred to the liquid nitrogen tank for storage. One cryotube from each batch of freezing was thawed periodically to check for viability.

8.3 Preliminary Test 1: Cell Seeding Density

From earlier experience with Saos-2 cells, it was observed that a suitable seeding density was required for cells to reach confluence. A low density of cells causes a drop in intercellular communication while at high densities, the cells become overcrowded and the environment becomes unfavourable. Work carried out on osteoprogenitor cells also showed that dispersed cells indicated better cell growth [161]. Hence, a test was carried out to find a suitable seeding density for cytotoxicity testing of the scaffolds.

Cells in the culture flask were first counted and found to have a density of 5.76×10^5 per mL. Cells were aliquoted in different volumes into the 24-well plate and media was added to make up 1mL in total. A control experiment using wells filled only with media was also set up. Table 8.1 summarises the experimental set up.

Table 8.1: Experimental set up for cell seeding density test

	Vol of aliquot (mL)	Vol of media (mL)	Cell density (cells/mL)
Set 1	0.05	0.95	0.288×10^5
Set 2	0.10	0.90	0.576×10^5
Set 3	0.20	0.80	1.15×10^5
Set 4	0.30	0.70	1.73×10^5
Set 5	0.40	0.60	2.30×10^5
Set 6	0.50	0.50	2.88×10^5
Set 7	0	1.00	0

Results and Discussion

The media in the wells was replaced with fresh media on day 4. Micrographs were taken on Day 1, Day 4, and Day 7 and the proliferation in the different sets were compared visually.

It was observed that even on Day 1, sets 5 and 6 were already highly confluent (Figure 8.6).

Figure 8.6: Day 1 results for Sets 1 to 7: (a) - (g)

On Day 4, it was observed that there were crystals in sets 1 and 2 (Figure 8.7(h)). At this stage, the sets were about 25% and 40% confluent respectively (Figure 8.7).

Figure 8.7: Day 4 results for Sets 1 to-7 (a)–(g) and crystal formation (h)

No crystals were observed on Day 7 in any of the sets (Figure 8.8).

Figure 8.8: Day 1 results for Sets 1 to 7: (a) - (g)

From the micrographs, it was observed that for experiments spanning a duration of 1 week, the seeding density should be approximately 1×10^5 cells per mL. For Set 1, there were many bright circular spots on day 7, which indicated cell death: Osteosarcoma, being osteoblast-like, requires a minimal density for proliferation. While cells in set 2 were about 75% confluent on day 7, the slow initial growth rate on day 4 might have had adverse effects on the cells. Cells in sets 4,5, and 6 reached 100% confluence before day 7 which meant that the seeding cell density was too high.

The appearance of the crystals on days 4 may be explained by the fact that Saos-2 cells are known to secrete bone inducing agents [152], and some of these compounds may crystallise at pH values below 7.

It was noted that cells tended to congregate in the centre of the well during seeding even though they were well suspended to prevent aggregation. This could be due to the surface profile of the multi-well plate. Specifications of the multi-well plate do not indicate its specific surface profile. Another possibility is that it is caused by surface tension in the solution.

8.4 Preliminary Test 2: Effect of ultra violet (UV) sterilisation on PCL

As PCL has a low melting point, sterilisation using autoclave is not feasible as temperatures in autoclave reach 120° Celsius, much higher than PCL's melting point of 60° Celsius. As a result, ethylene oxide (EtO) or ethanol is used to sterilise PCL. One concern, however, is that as these solvents are toxic, any residue left in the scaffolds will have undesirable effects on the cells. Hence, the feasibility of sterilising PCL using ultra violet (UV) rays was explored. It is also noted that SLS reaches high temperatures during sintering and if aseptic procedures were employed to collect the samples after fabrication, the samples may be sterile and can be directly used for seeding.

8.4.1 Experimental set up

Discs of 15mm diameter and 1.25mm thickness were fabricated using SLS. The scaffolds were retrieved with sterilised tweezers and immediately stored in sterile 50mL centrifuge cubes and transferred to the tissue culture room in sealed bags. The tubes were then cleaned with 70% ethanol and brought into the hood. The discs were placed in four separate petri dishes and exposed to the UV rays for 15 minutes and flipped to expose the reverse side to the UV rays. The UV rays had a wavelength of 254nm and an intensity of 400nW/m². The procedure was repeated with more discs such that separate sets were exposed to UV rays for 15 minutes, 30 minutes, and 60 minutes for each side.

The discs were placed in 24-well plates together with the non-UV-sterilised samples. Three controls were also included: wells with cells as well as media without PCL discs, and wells with media with unsterilised PCL discs and wells without cells or PCL discs.

8.4.2 Analysis of cell culture

The wells were checked for adhesion on day 1 using a light microscope. As PCL is opaque, the wells without PCL discs were used as reference. On day 4, the media was changed and replenished. Contamination detection tests and end point cell proliferation assay were carried out on day 7.

Contamination Detection

Contamination detection tests for bacteria and fungi were carried out using the media from various samples using protocol described in Section 8.2. All samples including the unsterilised discs tested negative for fungi and bacteria.

Proliferation Assay

Colorimetry is commonly used to approximate the proliferation of cells. The most widely used method involves the reduction of a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, commonly known as MTT. This salt is taken up by cells and metabolised into a coloured formazan product by mitochondrial enzyme activity in living cells. Hence, only live cells can be used in this method [162].

The kit used was Vybrant MTT Cell Proliferation Assay Kit (Invitrogen Catalogue No. V13154). To perform the assay, 5mg each of MTT was first dissolved in 1mL of sterile PBS. The solution was stirred to achieve complete mixing and particulates were removed by filter sterilisation. Prior to performing the assays, the media in the wells was removed and the discs were transferred to new wells. 400µL of media was added to each well followed by 100µL of 5mg/mL MTT solution. The well-plates were then incubated for 4

hours at 37°C. Figure 8.9 shows the results of the test. Areas with live cells are coloured and the intensity of the colour indicates the density of the cells.

Figure 8.9: Sterilised PCL discs (a) 60 minutes (b) 30 minutes (c) 15 minutes (d) Unsterilised sample (e) Control with no disc (f) Control with disc without cells (g) Blank control

From the results, it was observed that PCL discs generally had a larger area of cells (stained regions) and a higher density (dark stains) compared to the polystyrene control. In addition, the proliferation of cells seemed to be better in samples that have been exposed to UV for a shorter time.

Conventionally, the results can be quantified adding 500 μ L to each well, a solution of sodium dodecyl sulfate (SDS) in 0.01M hydrochloric acid (HCl) with a concentration of 0.1g/mL, and incubating the mixture overnight at room temperature. This step solubilises the formazan salt formed and releases the colour into the media. The media can then be transferred into 96-well plates and the concentration can be determined by optical density at 570nm using a spectrophotometer. However, in this experiment, it was observed that

when SDS-HCl was added, the resulting colour was absorbed into the PCL discs, making it difficult to quantify the results.

8.4.3 Analysis of material properties

From cell culture results, it was suspected that UV may have affected the surface or bulk material properties of PCL. A number of tests were thus carried out for further verification.

Raman Spectroscopy

Raman spectroscopy is used to study the vibrational or rotational modes in a system. It is useful in identifying the type of bonds present in a material as each chemical bond has a characteristic vibrational frequency without any damage to the samples. The raman spectrometer used in this experiment was Renishaw Raman 1000. Figure 8.10 shows the results from Raman spectroscopy of the samples.

Figure 8.10: Results from raman spectroscopy

It was seen from the graphs that all the samples had the same peaks and hence, similar waveforms. For samples that have been sterilised for 15 minutes (UVPCL15) and 60 minutes (UVPCL60), the background count was very high and caused a shift upwards in the waveform. However, the results might not be conclusive as background counts are due to environmental factors. Hence, the results are only be used for reference.

X-ray Diffraction (XRD)

In XRD, the diffraction pattern of x-rays through the lattice of the atoms of a crystal is used to understand the nature of the molecular structure. A non-destructive test, the pattern of diffraction peaks can be compared with those in material databases to identify the material being tested. Changes in position or peak width indicate differences in crystal size, purity and texture.

XRD follows Bragg's law:

$$n\lambda = 2d (\sin \theta) \quad (8.2)$$

where n is an integer, λ is the wavelength of x-rays, d is the spacing between the planes in the atomic lattice, and θ is the angle between the incident ray and the scattering planes

The XRD machine used was Phillip 1820 XRD system. The angle θ was varied from 10° to 80° . To minimise errors, it was ensured that the solid samples were placed flat. Subsequently, x-ray intensity was plotted against diffraction angle θ (Figure 8.11).

From the graphs in Figure 8.11, it was observed that positions of the peaks were similar in all samples. However, the width of the two prominent peaks (20° - 25°) were significantly

wider in the unsterilised samples as compared to UV-sterilised samples. Hence, it can be concluded that UV alters the crystallinity of PCL.

Figure 8.11: X-ray diffraction patterns of sterilized and non-sterilised samples

Thermogravimetric Analysis (TGA)

TGA is used to measure the change in the mass of a sample as it is heated up and degrades over time. TGA is commonly used to determine characteristics of polymers such as degradation temperatures and level of organic and inorganic components in materials. Perkin Elmer TGA Series 7 was used for this analysis.

For each test, a small piece of sample was broken from the discs approximately weighing about 5g. Samples were heated from 30⁰C to 600⁰C at 10⁰C/min in nitrogen and the weight loss in percentage was plotted as a function of temperature (Figure 8.12).

Figure 8.12: TGA results

From the results, two obvious differences between UV-sterilised and unsterilised samples can be noted. Firstly, onset temperature of degradation decreased with increase in UV exposure. Secondly, UV-sterilised samples behaved very differently towards the end of degradation between the temperatures of about 400-500° Celsius.

From the various tests carried out, it was seen that while UV-sterilised samples did not expressly improve cell proliferation or sterility of the samples but UV sterilisation altered the chemical properties of the material. As PCL is a biodegradable material, any changes in material properties may not be desirable. As long-term effects of such changes cannot be ascertained, it was concluded that UV sterilisation was not suitable for PCL.

8.5 Effect of microarchitecture on cell proliferation

To test the hypothesis that internal shapes of the scaffolds influence cell proliferation, three types of scaffold patterns were selected from the library: octahedron-tetrahedron, cuboctahedron-octahedron, and square prism. For each design, the strut length was set to 1.25mm with a strut diameter of 0.25mm. The discs were 15mm in diameter and 1.25mm in height. The different patterns are shown in Figure 8.13.

Figure 8.13: Different scaffold designs (a) Octahedron-tetrahedron (b) Cuboctahedron-octahedron (c) Square prism

It was noted that as the pores were much larger than the size of the cells, cells may fall through the gaps within the structures at initial cell seeding. As a result, another set of scaffolds was created with a solid base layer of 0.1mm for each design. Sets of solid PCL scaffolds and DuraformTM discs of the same dimensions were also fabricated for comparison purposes. Two other controls within the well plate, one with cells and one without cells were also set up.

Each scaffold (and the well for control) was seeded with 1×10^5 cells. On day 2, the scaffolds were transferred to new wells so that the proliferation of cells on scaffolds

would not be affected by those that have adhered to the bottom surface of the well. Media was changed every 3 days and MTT assay was carried out after 1 week, 2 weeks and 6 weeks in culture. Table 8.2 summarises the experimental set up.

Table 8.2: Experimental setup for investigation of effect of microarchitecture

Item	Name	Description
1	S1	Octahedron-Tetrahedron configuration without base
2	S2	Octahedron-Tetrahedron configuration with base
3	S3	Cuboactahedron-Octahedron configuration without base
4	S4	Cuboactahedron-Octahedron configuration with base
5	S5	Square prism configuration without base
6	S6	Square prism configuration with base
7	C1	Solid scaffold
8	C2	Duraform TM disc
9	C3	Polystyrene (well-plate)
10	C4	Blank control without cells

Note: All scaffolds were made of PCL unless otherwise stated

Observations

It was observed that PCL particles tend to rub off and aggregate at the bottom of the cell culture wells. Studies have been carried out to investigate the effect of nano-particles on cell function [163, 164] but none of these focused on PCL particle. Lu *et al* showed that micro HA particles do inhibit cell proliferation and cause damage to fibroblast cells placed in direct contact with the particles [164]. Hence, while PCL particles seemed to have no effects on the viability of the cells, tests can be carried out further to establish the influence of PCL particles on the cells.

Figures 8.14 and 8.15 show the scaffolds at 1 and 2 weeks. The MTT assay stains the cell populated areas a dark colour. It was observed that at the end of week 1, the control well was almost confluent. However, at the end of the 2nd week, while the proliferation increases in scaffolds (the colours were darker and occupy a larger area), in the polystyrene control, there was a smaller area of cells.

Figure 8.14: Week 1 results: (a)S1 (b)S2 (c)S2 (d)S4 (e)C3 (f)S5 (g)S6 (h)C1 (i)C2 (j)C4

Figure 8.15: Week 2 results: (a)S1 (b)S2 (c)S2 (d)S4 (e)C3 (f)S5 (g)S6 (h)C1 (i)C2 (j)C4

After 6 weeks, scaffolds were found to have very high cell content while the polystyrene control had no cells. In PCL solid discs and duraformTM solid discs, it was observed that the cells did not penetrate to the other side of the discs. This is due to lack of pores which restrict cell motility. Cell proliferation was highest for octahedron-tetrahedron scaffolds which exhibited darkest stains (Figure 8.16).

Figure 8.16: Top and bottom views of scaffolds after 6 weeks in culture

IX CONCLUSIONS AND FUTURE WORK

9.1 Conclusions

9.1.1 Development of Computer Aided System for Tissue Scaffolds

A prototype CAD system of structures based on convex polyhedral units has been developed and created for use with SLS for fabrication of TE scaffolds. The system is called the ***COMPUTER AIDED SYSTEM FOR TISSUE SCAFFOLDS*** or ***CASTS***.

CASTS consists of a basic library of units that can assemble uniform matrices of various shapes. Together with an algorithm which allows the designer to specify the unit cell and the required dimensions, the system is able to automatically generate a porous structure. Combined with imaging techniques, CASTS can generate 3-D models of implants with pre-designed architecture for patient-specific implants.

The main advantage of CASTS is the elimination of reliance on user skills unlike conventional techniques of scaffold fabrication. From a small range of basic units, many different scaffolds of different architecture and properties can be designed. The system interface of CASTS in Pro/ENGINEER is user friendly and allows complete transfer of knowledge between users without the need for complex user manuals.

9.1.2 Validation of CASTS using SLS

The successful fabrication of femur shape scaffold has proved the feasibility of applying CASTS. As noted in Section 5.2, one limitation was the presence of broken struts at the edges where the surface model intersects with the pre-generated scaffold structure.

However, this has minimal effect on the structural integrity of the scaffold when the unit cell sizes (measured in terms of strut length l) are small.

Duraform™ Polyamide scaffolds

Another problem faced was the removal of trapped powder in the scaffolds. A multi-step approach was taken to overcome this problem. First, scaffolds were fabricated with only one layer unit thickness. This serves a dual purpose; for in vitro experiments, thinner scaffolds are favoured. Machine parameters were examined and part bed temperature was varied to see its effects on amount of trapped powder in the scaffolds. The results showed that part bed temperature did influence the amount of powder trapped and this amount is reduced with a decrease in part bed temperature. Ultrasonic cleaning was used to further remove the trapped powder. Though there was no significant decrease in the amount of powder trapped, this method may be combined with another means of cleaning to increase the effectiveness.

Although Duraform™ is only a test material and cannot be used as actual scaffolds, the investigation gave insight to the possible problems which might occur during scaffold building and the plausible solutions to overcome them.

Biomaterial scaffolds

PEEK, being a bioinert material, must be combined with a bioactive material like HA to produce osteoconductive scaffolds. In this study, PEEK scaffolds are first fabricated for two reasons: as a test material before PEEK-HA biocomposite scaffolds are fabricated and also to act as a control in future in vitro and in vivo experiments. The parameter settings used produced good samples of both PEEK and PEEK-HA scaffolds though it is

not yet known whether the quality of the scaffolds can be improved by varying the parameters.

The results indicated that when the unit cell size is small, a lower energy density (e.g. a decrease in fill laser power) might produce better scaffolds; high energy densities create large thermal gradients which induce curling of specimens [117]. When the same intensity is used, there is curling at the edges of the samples and as a consequence, misalignment of the subsequent layers. However, the energy density cannot be too low as this has shown to reduce the strength of the scaffold in the Duraform™ samples.

Poly- ϵ -caprolactone Scaffolds

Fabrication poly- ϵ -caprolactone scaffolds were found to be more viable than PEEK or PEEK-HA biocomposite scaffolds. Not only was there consistency in structure, there was little problem with powder trapped within the scaffolds due to the elastic nature of sintered PCL. As a result, PCL scaffolds were used to carry out mechanical characterisation and in vitro studies.

Compression tests done on PCL samples showed that mechanical properties of the scaffolds are dependent on the design of the unit cell as well as the porosity of the scaffold. In vitro testing using PCL scaffolds showed promising results. MTT stains showed that the cells have penetrated into the scaffolds and also at different rates for different scaffold designs.

Hence, CASTS is a viable system for generating and producing scaffolds for tissue engineering applications.

9.2 Future recommendations

9.2.1 Further work on CASTS

From this project, it has been shown that CASTS is a viable method of generating and producing scaffolds using at least one RP technology, Selective Laser Sintering. The next step would be to widen the range of materials using CASTS by either experimenting with more materials using SLS itself or by employing different RP systems to fabricate scaffold designed using CASTS. Already, more biomaterials are ready for use with the SLS. These materials include PVA and PVA-HA composites [98] as well as PCL-HA composites [146]. Currently, scaffolds are being tested out on other systems such as the Fused Deposition Modeling system which has been successfully used to build non-polyhedral scaffolds using PCL and PCL-HA composites [165].

CASTS can be further adapted to generate scaffolds with porosity gradient. Organs are made up of different types of cells and hence, have different requirements in terms of pore sizes and porosity. Currently, CASTS is capable of generating scaffolds with different porosities. However, in order to generate a scaffold with varying porosity across the volume, post-processing in another software is needed. It will be more efficient to incorporate an algorithm for generating scaffolds with porosity gradients.

9.2.2 Further characterisation

This thesis presents data on three of the unit cells of the parametric library. Characterisation needs to be done for all unit cell types in order to build a comprehensive database. As mentioned in Chapter 2, properties of scaffolds, chemical and mechanical, are closely related to cell attachment, growth and proliferation. Having a complete database of these properties will make application much better and more efficient.

Further studies can be done to investigate the influence of polyhedral shapes and sizes on different cell types in relation with their mechanical and material properties. As it is not feasible to fabricate tensile test samples, indentation testing can be used to measure the Poisson ratio of sintered PCL or other new materials.

9.2.3 *In vitro* and *in vivo* applications

The use of osteosarcoma for *in vitro* studies, while a good indicator of cell cytotoxicity and proliferation, is limited. Firstly, while the strain used has been proven to behave similarly to normal osteoblasts, immortal cells tend to be sturdier. Furthermore, sarcoma cells cannot be used in clinical studies as they may still prove to be cancerous. Use of specialised cells such as osteoblasts are also not recommended as many tissues are made up of a variety of cell types and specialised cells usually lack the ability to de-differentiate and hence, grow into other types of cells. Hence, the potential of tissue engineering lies in harvesting stem cells and seeding them into scaffolds to grow viable organs. Research has shown that even stem cells from specific organs maybe capable of generating functional tissue [166, 167]. However, methods used may need to be adapted

as Saos-2 cells have been found to use different mechanisms from that of stem cells to adhere to biomaterials such as Hydroxyapatite [168].

Furthermore, to enhance the viability of the scaffolds, the surfaces maybe coated with integrins to encourage binding of molecules such as fibronectin and vitronectin which are constituents of ECM of many tissues and are also found abundantly in the human serum [168]; osteoblastic cell attachment tends to be supported by materials that absorb higher quantities of vitronectin [169, 170].

Also, as this work has shown that ultra violet rays are not suitable for sterilisation of PCL scaffolds, sterilisation through gamma radiation can be explored.

9.3 Publications

Journal Papers

1. Naing MW, Chua CK, Leong KF, Wang YQ. Fabrication of customised scaffolds using Computer-aided design and Rapid Prototyping techniques. *Rapid Prototyping Journal*. Accepted for publication, Mar 2005.
2. Tan KH, Chua CK, Leong KF, Naing MW, Cheah CM, Cheang P. Fabrication and Characterisation of 3D Polyetheretherketone/hydroxyapatite Biocomposite Scaffolds using Laser Sintering. *ICME: Part H. Journal of Engineering in Medicine*, Vol 219, pp 183-194, 2004.
3. Cheah CM, Chua CK, Leong KF, Cheong CH, Naing MW. An automatic algorithm for generating complex polyhedral scaffold structures for tissue engineering. *Tissue Engineering* Vol 10 No. 2, pp 595-610, 2004.

Conference Papers

1. Naing MW, Chua CK, Leong KF. Integrated system for Tissue Engineering Scaffolds (Invited paper). Presented at Materialise Mimics User Group Conference, 12th International Conference on Biomedical Engineering, Singapore, Dec 2005.
2. Naing MW, Chua CK, Leong KF. Customised tissue scaffolds using integrated CAD system (Awarded 2nd prize for Best Paper). Presented at the *3rd International Symposium on Advanced Biomaterials and Biomechanics*, Montreal, Canada, Apr 2005.
3. Naing MW, Chua CK, Leong KF. Novel Computer-Aided Fabrication of Cellular scaffolds for Tissue Engineering, *Proceedings of International Conference for Epithelial Technologies and Tissue Engineering*, Singapore, Dec 2003.

4. Chua CK, Leong KF, Wee WE, Cheah CM, Sitthiseriparatip K, Naing MW. Production Of Custom Cranioplasty Implants By Rapid Prototyping. *Proceedings of International Conference for Virtual and Rapid Prototyping*, Leira, Portugal, Oct 2003.
5. Chua CK, Naing MW, Leong KF, Cheah CM. Novel Method for producing polyhedral scaffolds in tissue engineering. *Proceedings of International Conference for Virtual and Rapid Prototyping*, Leira, Portugal, Oct 2003.

Papers in Progress

1. Naing MW, Chua CK, Leong KF, Sudarmadji N. CAD designed scaffolds with controlled porosity gradient. To submit to Biotechnology and Bioengineering.
2. Naing MW, Chua CK, Leong KF. CAD designed Polycaprolactone scaffolds fabricated using Selective Laser Sintering. To submit to Tissue Engineering.
3. Naing MW, Chua CK, Leong KF. Effect of ultra violet exposure on sintered polycaprolactone scaffolds. To submit to Biomaterials.

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