Novel indirect approach to fabricating tissue engineering scaffolds

Tan, Jiayong

2011

Tan, J. Y. (2011). Novel indirect approach to fabricating tissue engineering scaffolds.
Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/44876>

<https://doi.org/10.32657/10356/44876>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**A NOVEL INDIRECT APPROACH TO FABRICATING
TISSUE ENGINEERING SCAFFOLDS**

TAN JIAYONG

SCHOOL OF MECHANICAL AND AEROSPACE ENGINEERING

2011

**A NOVEL INDIRECT APPROACH TO FABRICATING
TISSUE ENGINEERING SCAFFOLDS**

TAN JIAYONG

School of Mechanical and Aerospace Engineering

A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2011

Abstract

One of the key elements involved in the tissue engineering process is the scaffold which serves as a three-dimensional (3D) template for cell organization and tissue development. A scaffold can be expected to meet a wide range of macro- and micro-structural requirements due to the diverse nature of tissues. The approach to fabricate the scaffold then, becomes an important consideration for tissue engineers since the overall architecture of most scaffolds is largely determined by its fabrication process. With the increasing number of studies demonstrating the profound influences of scaffold pore architecture on cell behavior and overall tissue growth, a scaffold fabrication method with sufficient architectural control becomes imperative. Yet, the conventional methods of scaffold fabrication such as solvent casting, gas foaming, phase separation and etc. are rather inadequate in providing rigorous control over the entire scaffold pore architecture. More advance methods of fabrication such as rapid prototyping (RP) technologies, although having a higher degree of control over certain aspects of scaffold structure, are still unable to reliably produce micron-sized features due to resolution limitations. In the present study, a scaffold fabrication technique with the capability of offering users sufficient degree of macro- and micro-architectural control is proposed. This thesis describes the developmental process of an inverse fabrication approach that involves the application of RP technology in conjunction with a microsphere leaching process to produce highly and uniformly porous scaffolds with complex channel architectures. The concept behind this fabrication methodology is an inverse strategy in which the entire internal structure of the scaffold is shaped using a lost-mold technique. This mold, which is termed as the inverse mold throughout the chapters, is produced using parts built from a liquid-based RP system as well as polystyrene microspheres.

As part of the inverse scaffold fabrication process, a liquid-based RP system which operates on the PolyJet™ technology was employed to produce the RP part. The shape, dimension, distribution, configuration as well as the orientation of the channels can be easily designed and controlled using the proposed inverse fabrication method. To better understand the fundamentals of the PolyJet™ process and the difference in the two printing modes, an analytical model was established to correlate the operating

parameters of the RP system to the dimensions of the fabricated part. The RP part is eventually filled with microspheres that are aligned in a hexagonally close-packed (HCP) structure through the use of an ultrasound agitation method. The fabricated RP part and polystyrene microspheres are eventually fused together to form the negative replica of the entire scaffold architecture. An analytical model was also established to correlate the sintering time interval to the neck radius of fused microspheres and overall packing density of the inverse mold. These parameters correspond to the interconnection size between pores and the porosity of the final scaffold.

Preliminary *in vitro* characterization studies were also performed to assess the biocompatibility of the fabricated scaffolds. On the basis of the cytotoxicity assessment results, it was clear that the multiple steps and materials involved in the inverse fabrication process did not induce any toxicity in the scaffold. Cell culture experiments with fibroblast cells showed that cell proliferation is higher in scaffolds with a larger pore interconnection size. By combining RP fabricated molds with microspheres, the proposed inverse fabrication method in this study has provided a promising alternative way to prepare scaffolds with tailored pore architectures that can satisfy specific requirements of the tissue e.g., porosity, pore size, pore interconnection size, etc.

Acknowledgements

The author would like to take this opportunity to acknowledge the contributions from a number of people for this project. The realization of the project would not have been possible without the advice and assistance from them.

The author wishes to express his sincere gratitude and appreciation to A/P Chua Chee Kai and A/P Leong Kah Fai for their invaluable advice and motivation throughout the project. Appreciation is also extended to A/P Chian Kern Sim from the School of Mechanical & Aerospace Engineering, NTU, and Mr Timothy Tan and Dr Peter Lee from DNA Center, NIE, for their guidance in the project.

This report would not have been possible without the assistance of the technical staff from Advanced Design and Modeling Laboratory, Tissue Engineering Laboratory and Materials Laboratory in School of Mechanical and Aerospace Engineering as well as DNA Center, National Institute of Education.

Special thanks to the fellow graduate students from the same laboratory for their support and friendship. Last but not least, the author would like to convey his appreciation to his family for their unconditional love and support.

Table of Contents

<u>Abstract.....</u>	<u>i</u>
<u>Acknowledgements.....</u>	<u>iii</u>
<u>Table of Contents.....</u>	<u>iv</u>
<u>List of Figures.....</u>	<u>viii</u>
<u>List of Tables.....</u>	<u>xiv</u>
<u>Nomenclature.....</u>	<u>xv</u>
<u>Chapter 1 – INTRODUCTION.....</u>	<u>1</u>
<u>1.1 Background.....</u>	<u>1</u>
<u>1.2 Motivation.....</u>	<u>3</u>
<u>1.3 Objectives.....</u>	<u>8</u>
<u>1.4 Scope.....</u>	<u>9</u>
<u>1.5 Organization of Report.....</u>	<u>10</u>
<u>Chapter 2 – LITERATURE REVIEW.....</u>	<u>12</u>
<u>2.1 Essential Properties of TE Scaffolds.....</u>	<u>13</u>
<u>2.1.1 Scaffold Porosity.....</u>	<u>13</u>
<u>2.1.2 Optimal Pore Size.....</u>	<u>14</u>
<u>2.1.3 Pore Interconnectivity.....</u>	<u>16</u>
<u>2.1.4 Scaffold Degradation.....</u>	<u>17</u>
<u>2.1.5 Surface Properties.....</u>	<u>19</u>
<u>2.1.6 Mechanical Strength.....</u>	<u>20</u>
<u>2.2 Scaffold Materials.....</u>	<u>21</u>
<u>2.2.1 Biological Materials.....</u>	<u>22</u>
<u>2.2.2 Synthetic Materials.....</u>	<u>22</u>
<u>2.3 Scaffold Fabrication Methods.....</u>	<u>23</u>
<u>2.3.1 Conventional Fabrication Methods</u>	<u>24</u>
<u>2.3.1.1 Gas Foaming</u>	<u>24</u>
<u>2.3.1.2 Solvent Casting/Particulate Leaching</u>	<u>25</u>
<u>2.3.1.3 Emulsion/Freeze Drying.....</u>	<u>27</u>
<u>2.3.1.4 Thermally-Induced Phase Separation.....</u>	<u>29</u>
<u>2.3.1.5 Reverse Templating.....</u>	<u>31</u>
<u>2.3.2 Rapid Prototyping Techniques</u>	<u>32</u>
<u>2.3.2.1 Fused Deposition Modeling (FDM).....</u>	<u>33</u>
<u>2.3.2.2 3D Bioplotting.....</u>	<u>35</u>
<u>2.3.2.3 3-Dimensional Printing (3DP).....</u>	<u>35</u>
<u>2.3.2.4 Selective Laser Sintering (SLS).....</u>	<u>36</u>
<u>2.3.2.5 ModelMaker II (MMII).....</u>	<u>37</u>
<u>2.3.2.6 Stereolithography (SLA).....</u>	<u>39</u>
<u>2.3.3 Electrospinning</u>	<u>40</u>
<u>2.4 Challenges in Scaffold Fabrication.....</u>	<u>41</u>
<u>2.4.1 Limitations of Conventional Fabrication Methods</u>	<u>42</u>
<u>2.4.2 Resolution and Material Limits of RP Techniques.....</u>	<u>44</u>

2.5 Summary.....	47
Chapter 3 – INVERSE SCAFFOLD FABRICATION PART I: RAPID	
PROTOTYPING PROCESS.....	49
3.1 Background on Inkjet Technology and Three Dimensional Printing.....	49
3.1.1 Bubble Jet Printing.....	50
3.1.2 Piezoelectric Ejection.....	50
3.1.3 Piezoelectric Technology in Rapid Prototyping.....	51
3.2 Objet's PolyJet™ Printing Process.....	52
3.2.1 Printheads.....	53
3.2.2 Printing Sequence.....	55
3.2.3 Overview of 3D Model Fabrication.....	56
3.2.4 Kinetics of Droplet Spreading	57
3.2.5 Line Formation	63
3.2.6 Layer Thickness	66
3.2.7 Section Summary	71
3.3 Dimensional Accuracy.....	72
3.4 Chapter Summary.....	76
Chapter 4 – INVERSE SCAFFOLD FABRICATION PART II:	
MICROSPHERE PREPARATION.....	77
4.1 Requirements for the Micro-Particles.....	77
4.1.1 Solvent and Scaffold Material Compatibility.....	78
4.1.2 Uniform Particle Shape and Size.....	78
4.1.3 Ease of Production and Availability in Different Sizes.....	78
4.2 Inverse Mold Fabrication Outline.....	79
4.3 Microsphere Preparation.....	79
4.3.1 Microsphere Material.....	79
4.3.2 Microsphere Fabrication.....	80
4.3.3 Separation and Storage.....	81
4.3.3.1 Microsphere Separation.....	82
4.3.3.2 Handling and Storage.....	84
4.4 Microsphere Packing.....	86
4.4.1 Method of Assembly.....	86
4.4.2 Stacking of Microsphere Layers	93
4.4.3 Microsphere Assembly in RP Mold	95
4.5 Mold Sintering.....	96
4.6 Chapter Summary.....	99
Chapter 5 – THEORETICAL ANALYSIS OF SCAFFOLD STRUCTURE ...	100
5.1 Microsphere Sintering Process.....	100
5.1.1 Phases of Sintering	101
5.1.2 Maximum Neck Length	102
5.1.3 Driving Forces of Necking	103
5.2 Theoretical Analysis of Pore Interconnection Size and Scaffold Porosity....	104
5.2.1 Neck Radius.....	105
5.2.2 Local Porosity.....	108
5.2.2.1 Unit Cell Concept.....	110
5.2.2.2 Volume of a Unit Cell.....	114
5.2.3 Compensation for Local Porosity.....	116
5.2.3.1 Imperfect Stacking.....	117

5.2.3.2 Non-Contact Points of Individual Microspheres.....	119
5.2.4 Global Porosity.....	121
5.3 Chapter Summary.....	122
Chapter 6 – EXPERIMENTAL PROCEDURES	124
6.1 Scaffold Fabrication.....	124
6.1.1 Mold Preparation.....	125
6.1.1.1 RP Mold Design.....	126
6.1.1.2 RP Mold Fabrication.....	128
6.1.1.3 Second Mold Fabrication.....	129
6.1.1.4 Inverse Mold Fabrication.....	130
6.1.1.5 Mold Preparation for Local Porosity Test.....	131
6.1.2 Mold Casting.....	132
6.1.3 Mold Removal.....	134
6.1.4 Scaffold Drying.....	135
6.2 Scaffold Characterization.....	136
6.2.1 Morphology of Scaffold Structure	136
6.2.2 Porosity Measurement	136
6.2.3 Measurement of Number of Necks on Microspheres	137
6.3 Preliminary In Vitro Characterization.....	138
6.3.1 Cell Culture Process	138
6.3.1.1 Cell Culture Reagents and Equipments	139
6.3.1.2 Cell Thawing	140
6.3.1.3 Cell Passaging	141
6.3.1.4 Cell Freezing	142
6.3.1.5 Cell Counting	142
6.3.1.6 DNA Cell Quantification	143
6.3.2 Cytotoxicity Assessment of Scaffolds.....	145
6.3.2.1 Extract Dilution Cytotoxicity Test	145
6.3.2.2 Extract Preparation	145
6.3.2.3 Cytotoxicity Test Procedure.....	146
6.3.3 Cell Proliferation Assay.....	146
6.3.3.1 Scaffold Samples.....	146
6.3.3.2 Scaffold Sterilization.....	147
6.3.3.3 Cell Seeding.....	148
6.3.3.4 Cell Proliferation Analysis.....	148
6.3.3.5 Statistical Analysis.....	149
Chapter 7 – RESULTS AND DISCUSSIONS	150
7.1 Inverse Scaffold Fabrication Procedure.....	150
7.1.1 Production of Inverse Molds	150
7.1.2 The Procedure Concerning Mold Removal and Crosslinking	152
7.1.2.1 Crosslinking Treatment before Mold Removal.....	153
7.1.2.2 Crosslinking Treatment after Mold Removal.....	156
7.1.3 Scaffold Drying	158
7.1.4 Effect of Ultrasound Agitation on Pore Structure.....	160
7.1.5 Summary of the Inverse Scaffold Fabrication Method.....	160
7.2 Scaffold Characterization.....	161
7.2.1 Pore Interconnection Size	161
7.2.2 Frenkel’s Model Predictions	163
7.2.3 Scaffold Porosity	167

7.2.3.1 Global vs Local Porosity	168
7.2.3.2 Local Porosity: The Effect of Ultrasound Agitation	170
7.2.4 Porosity Model Predictions with Compensation	173
7.2.4.1 Compensation via Non-Contacting Points	173
7.2.4.2 Compensation via Stacking Defects	174
7.3 Preliminary In Vitro Characterization.....	177
7.3.1 Scaffold Cytotoxicity Test.....	177
7.3.2 Scaffold proliferation test.....	180
7.3.2.1 Cell Proliferation on Scaffolds prepared via Microsphere Templates	181
7.3.2.2 Results of Scaffolds Prepared from both types of Inverse Molds.....	183
Chapter 8 – CONCLUSIONS AND FUTURE WORK	187
8.1 Conclusions.....	187
8.1.1 The Development of the Inverse Scaffold Fabrication Method.....	187
8.1.2 Theoretical Modeling of Scaffold Structure.....	189
8.1.3 Preliminary in vitro Characterization.....	190
8.2 Future Work.....	190
8.2.1 Improving the Design of the Inverse Mold.	190
8.2.2 Further Characterization on Scaffolds produced via Inverse Fabrication Method.....	191
8.2.3 In Depth In Vitro and In Vivo Studies	192
8.3 List of Publications.....	193
8.3.1 Journal Papers	193
8.3.2 Conference Papers	193
References.....	195
Appendix.....	196
Plots of measured widths in various orientations.....	00000000
Plots of measured widths in various orientations.....	A1-A4
Tabulation of p values.....	B1
Tabulation of t values	B2
Tabulation of p values for Cytotoxicity test (Figure 7.25).....	B3
Tabulation of p values for Figure 7.27 and 7.28.....	B3

List of Figures

Figure 1.1: Illustration of Indirect scaffold fabrication using RP technique.....	6
Figure 2.2: Scaffold-guided tissue regeneration.....	12
Figure 2.3: Porous structure of PLLA scaffold	14
Figure 2.4: Cross-sectional organization of tissue in the human esophagus: Mucosa (M) consisting of epithelium (E) and the muscularis mucosa (MM), the submucosa (SM) and muscularis externa showing the inner circular (IC) and outer longitudinal (OL) muscle layer	15
Figure 2.5: Cell integrin-ligand mediated adhesion.....	20
Figure 2.6: PCL scaffolds fabricated via gas foaming	25
Figure 2.7: Fabrication of scaffold using the porogen leaching method.....	26
Figure 2.8: Scaffolds illustrating pores with limited interconnection to other pores as well as closed pores.....	27
Figure 2.9: Illustration of the emulsion/freeze drying approach.....	28
Figure 2.10: Illustration showing the solvent phase and polymer phase after crystallization process.....	30
Figure 2.11: Illustration of the reverse templating process.....	31
Figure 2.12: Schematic diagram of FDM process and FDM parameters, including slice thickness(ST), road width (RW), and raster gap (RG)	34
Figure 2.13: Scaffold with porosity gradient in radial direction fabricated by FDM	35
Figure 2.14: Indirect 3DP process showing the mold and the final PLGA scaffold	36
Figure 2.15: A schematic diagram of MMH process	38
Figure 2.16: ProtoSupport™ Mold with struts branching in x, y and z directions	38
Figure 2.17: Schematic diagram of SLA process.....	39
Figure 2.18: A small section of the scaffold illustrating pores with only a single opening.....	43
Figure 2.19: FDM fabricated PP-TCP composite scaffolds	44
Figure 2.20: PCL scaffolds with interconnected channels fabricated using indirect SLA process	45

Figure 2.21: Collagen scaffolds with intricate channel features fabricated from MMII mold	46
Figure 3.22: Bubble jet printing	50
Figure 3.23: Piezoelectric printing	51
Figure 3.24: Schematic representation of droplet-based fabrication process.....	52
Figure 3.25: Part divided into several layers each depicting the cross section of the part.....	53
Figure 3.26: UV lamps attached to the printhead curing the layer as it traverses along the build tray.....	54
Figure 3.27: Several parallel lines form the image of the cross section of the part	54
Figure 3.28: Alternate lines printed on the substrate during the forward pass...	55
Figure 3.29: Alternate line printing scheme.....	56
Figure 3.30: Overview of fabrication process of a 3D model.....	57
Figure 3.31: From droplet impact (state1) to maximum droplet spread (state2)	58
Figure 3.32: Individual photopolymer droplets (state 2) collected from the Objet printer A) Top view (80X), B) Side view (500X).....	59
Figure 3.33: Spreading into a cylindrical disk of thickness, h.....	62
Figure 3.34: Illustration of line formation involving overlapping droplets.....	64
Figure 3.35: Illustration on the overlapping of cylindrical discs in quality print mode.....	65
Figure 3.36: Cross sectional area of a printed line.....	67
Figure 3.37: Formation of uneven layer thickness ($> 2W$).....	68
Figure 3.38: Illustration of the formation of a single layer (cross sectional view) Blue: lines printed during forward pass. Red: lines printed during the return pass.....	68
Figure 3.39: Profile of A) alternate lines, B) one layer obtained using a contact profilometer.....	69
Figure 3.40: A) lines printed in quality mode, B) lines printed in speed mode....	69
Figure 3.41: Geometrical analysis of layer thickness.....	70
Figure 3.42: Part to be printed for measurement.....	73
Figure 3.43: Layout of the printed parts for measurement.....	73
Figure 3.44: Top view of the fabricated strip (oriented in the x-direction) showing the rectangular feature that was designed at 550 μm	75

Figure 3.45: Plots of measured widths in various orientations. A) x- direction, B y- direction, C) x-y direction, D) y-x direction.....	75
Figure 4.46: The various stages of inverse mold fabrication.....	79
Figure 4.47: Polystyrene microsphere fabrication using oil-in-water emulsion technique.....	81
Figure 4.48: Polystyrene microspheres of random sizes (20X magnification, scale bar: 100 μm).....	82
Figure 4.49: The sieving process.....	83
Figure 4.50: Polystyrene microspheres with an average diameter of 88 μm	83
Figure 4.51: 53 μm polystyrene microspheres in water with aggregation (indicated by the circles).....	85
Figure 4.52: 53 μm polystyrene microspheres in IPA.....	86
Figure 4.53: Microspheres in Hexagonal closed packed arrangement and resulting scaffold.....	87
Figure 4.54: The evaporation-induced self assembly process.....	88
Figure 4.55: Arrangment of 100 μm polystyrene microspheres after evaporation of IPA with no agitation (scale: 500 μm).....	89
Figure 4.56: Methods of providing agitation to microsphere suspension to induce assembly: A) orbital shaker method, B) ultrasound vibration method.....	90
Figure 4.57: Arrangment of 100 μm polystyrene microspheres in IPA using the orbital shaker method (scale bar: 200 μm).....	91
Figure 4.58: Arrangment of 100 μm polystyrene microspheres in IPA subjected to ultrasound agitation.....	92
Figure 4.59: Demonstration of self stacking during assembly of 100 μm polystyrene microspheres.....	94
Figure 4.60: The effect of glass thickness on stacking thickness. A) 1, B) 2 and C) 3 glass slides.....	95
Figure 4.61: Experimental setup for dispensing the microspheres into the RP mold.....	96
Figure 4.62: A) The fabricated Inverse mold (comprising of the sintered RP mold and the microspheres), B) image of the assembly of microspheres using a feeding rate of 200 μl per 30 seconds and, C) feeding rate of 50 μl per minute.....	97
Figure 4.63: A 3D view of the assembly of microspheres showing an ordered HCP alignment in every layer.....	98

Figure 5.64: The 3 phases of the sintering process.....	101
Figure 5.65: Illustration of neck length A) before sintering and, B) after the final phase of sintering.....	102
Figure 5.66: Alignment of microspheres at maximum necking length in a 3D view.....	103
Figure 5.67: Numerical simulation of the necking process of 2 spheres showing the increase in neck radius along with the decrease in surface curvature at the neck region and the eventual decrease in total surface area of the material system over time	104
Figure 5.68: Sintering of 88 μm polystyrene microspheres for 32 hours.....	106
Figure 5.69: The necking of two spheres at $t = 0$ and $t = t$	107
Figure 5.70: Illustration of local porosity of a scaffold.....	110
Figure 5.71: 2D illustration of a unit cell (hexagon).....	111
Figure 5.72: Analysis of a 3D HCP structure A) 3D isometric view, B) Side view of the two regions of a single microsphere layer.....	111
Figure 5.73: A) 3D illustration of a regular tetrahedron, B) dimensions of the tetrahedron.....	112
Figure 5.74: Illustration of the change in unit cell over time.....	113
Figure 5.75: Geometry of 2 necked microspheres.....	113
Figure 5.76: Geometry of the shaded plane from figure 5.10b.....	114
Figure 5.77: Unit cell representation of a HCP microsphere assembled structure	115
Figure 5.78: SEM image of aligned 88 μm microspheres showing stacking faults (indicated by arrows).....	117
Figure 5.79: Illustration of a simple stacking defect.....	118
Figure 5.80: SEM image of HCP aligned 88 μm polystyrene microspheres (Arrows represent locations where there are no necking between microspheres)	120
Figure 5.81: 2D illustration for volume compensation of a non-contacting point of a microsphere.....	120
Figure 6.82: The various processes involved in the inverse scaffold fabrication method.....	125
Figure 6.83: Illustration of fabricating the second mold.....	126
Figure 6.84: CAD drawing of RP mold with 3D, top and side view.....	128

Figure 6.85: Objet studio software.....	129
Figure 6.86: Fabrication of the second mold: A) RP mold placed in oven, B) RP mold filled with LDPE pellets, C) LDPE pellets melted at 130 °C, D) Melted LDPE casted into the RP mold.....	130
Figure 6.87: Ultrasound bath setup for microsphere assembly.....	131
Figure 6.88: Ultrasound setup for microsphere template fabrication.....	132
Figure 6.89: Scaffold material casting using gelatin type B solution with A) 0% ethanol and, B) 40% ethanol.....	134
Figure 6.90: Polystyrene microsphere showing the neck regions (see arrows). .	138
Figure 6.91: Process flow of cell culture.....	139
Figure 6.92: Layer of fibroblast cells A) near-confluent layer of cells with spaces between individual cells, B) confluent monolayer of cells.....	141
Figure 6.93: a) Haemocytometer, B) Haemocytometer counting grids.....	143
Figure 7.94: Illustration of the RP component (A) Computer Aided Design (CAD) drawing of RP mold, (B) Transparent view of CAD drawing showing the internal interconnected channels, (C) 3D view of fabricated RP mold, (D) Top View of fabricated RP mold, (E) 3D view of LDPE mold, (F) Top view of LDPE mold.....	151
Figure 7.95: A) struts of the LDPE mold designed at 600 μm , B) channels of the actual gelatin scaffold.....	152
Figure 7.96: Inverse mold (LDPE mold filled with polystyrene microspheres).	152
Figure 7.97: Pore structure of scaffold fabricated using an inverse mold without struts. A) X 30, B) X 100.....	154
Figure 7.98: Gelatin scaffold fabricated via the inverse fabrication process. (A) gelatin scaffold immersed in ethanol, (B) SEM image of scaffold pore structure	155
Figure 7.99: Gelatin scaffolds that were crosslinked after mold removal with regions of non-spherical pores (see circled area).....	156
Figure 7.100: Illustration on the importance of crosslinking treatment before the mold removal process.....	157
Figure 7.101: Pore structure of gelatin scaffolds that were dried using A) freeze drying, B) critical point drying.....	159
Figure 7.102: Critical point drying (blue arrow) goes beyond the supercritical point of the working fluid in order to avoid the direct liquid-gas transition seen	

in oven drying (green arrow) and also the solid-gas transition (red arrow) in freeze drying	159
Figure 7.103: Pore structure of scaffold fabricated via inverse molds prepared without ultrasound agitation.....	160
Figure 7.104: Pore morphology of gelatin scaffolds that were created from inverse molds sintered at A) 1 hr, B) 8 hrs, C) 16 hrs, D) 24 hrs, E) 32 hrs, F) 40 hrs.....	162
Figure 7.105: Microsphere structure sintered for 48 hours illustrating a high degree of necking with little or no space between individual microspheres (see insert for closer magnification).....	163
Figure 7.106: Graph of pore interconnection size plotted against sintering time	164
Figure 7.107: Theoretical predictions from Frenkel's model vs experimental data	165
Figure 7.108: Plot showing the theoretical predictions from Frenkel's model with correction factor and experimental data.....	167
Figure 7.109: Experimental porosity plotted against sintering time (n=6).....	168
Figure 7.110: Comparison between experimental global and local porosity.....	169
Figure 7.111: Illustration on the amount of scaffold material taken up by a channel.....	170
Figure 7.112: Porosity comparison between scaffolds fabricated from microsphere templates with and without ultrasound agitation (n=6).....	171
Figure 7.113: Microsphere template assembled A) with ultrasound agitation and, B) without ultrasound agitation.....	172
Figure 7.114: Hexagonal arrangement of 88 μm polystyrene microspheres.....	173
Figure 7.115: Comparison of experimental porosity with theoretical predictions	174
Figure 7.116: Comparison of theoretical global porosity values and experimental data.....	176
Figure 7.117: Cell calibration curve for fibroblasts.....	178
Figure 7.118: Cytotoxicity assessment results: Number of fibroblasts cultured in fluid extracts derived from scaffolds relative to negative and positive controls (n=5).....	179

Figure 7.119: Microscopic images (20X) of cultured fibroblasts in 24-well plates and trypan blue staining respectively: (A, D) negative control, (B, E) positive control, (C, F) extracts derived from scaffolds.....	180
Figure 7.120: Cell proliferation on gelatin scaffolds (GS2, GS16, GS32) on the 1st, 5th, 10th, and 14th day of culture (n=5).....	182
Figure 7.121: Cell proliferation on gelatin scaffolds (GS2, GS16, GS32, GSC, CS) on the 1st, 5th, 10th, and 14th day of culture (n=5).....	184
Figure 7.122: The pore structure of control scaffolds showing irregular pore structure.....	185
Figure 8.123: Example of mold design for the multilayered esophageal tissue..	191

List of Tables

Table 3.1: Manufacturer's printer and material specifications.....	65
Table 3.2: Summary of part measurements.....	74
Table 5.3: Scaffolds and approaches.....	105
Table 6.4: Recommended ethanol concentrations.....	133
Table 6.5: Process routes for mold removal.....	135
Table 6.6: Cell dilution series.....	144
Table 7.7: Inverse scaffold fabrication procedure.....	161
Table 7.8: Co-relation between sintering time and interconnection size.....	163
Table 7.9: Calculated data of Frenkel's model with correction factor.....	166
Table 7.10: Tabulated data of lcc and Unit cell volume.....	168
Table 7.11: Porosity compensation via non-contact points.....	174
Table 7.12: Porosity compensation via stacking defects.....	175
Table 7.13: Comparison of theoretical local porosity and global porosity.....	177

Nomenclature

β	Spreading Ratio
d	Diameter of Droplet before impact
D_{max}	Diameter of Droplet at maximum spread
E_{k1}	kinetic energy at the instance of impact
E_{s1}	Surface energy of the droplet before impact
E_{k2}	kinetic energy at maximum spread
E_{s2}	surface energy of the droplet in contact with the surrounding vapor at maximum spread
E_{s2}	Interfacial energy between the spreaded drop and the solid substrate
E_{V2}	Energy loss through viscous dissipation
h	The height of the droplet at maximum spread
ρ	Density of the droplet
U	Impact velocity of the droplet
γ_{LV}	Surface tension at the liquid/vapor interface
γ_{SL}	Surface tension at the substrate/liquid interface
γ_{SV}	Surface tension at the substrate/vapor interface
ϕ	Viscous dissipation function
V_{drop}	Volume of the droplet
t_c	Time taken for the droplet to reach maximum spread
δ	Boundary Layer thickness
Re	Reynolds Number
μ	Viscosity of droplet
V_R	Velocity at the edge of spreading drop
We	Weber's Number
S	Scanning speed of printhead
f	Droplet frequency
C	Droplet center to center separation distance
N_{drop}	Total number of droplets
A	Cross-sectional area of line
L	Length of line

W	Line width
h_L	Line thickness
D_L	Line to line separation distance
A_{gap}	Cross sectional gap area
h_t	Layer thickness
V_L	The total volume of the neighboring droplets that are enclosed within the physical boundary of an individual spread droplet
d_L	The new drop diameter based on V_L
V_{dep}	The total volume of a printed line
y	Neck radius
r	Microsphere radius
t	Sintering time interval
k	Constant which depends on material properties such as surface tension, viscosity, modulus etc
η	Viscosity of microspheres during sintering
γ	Surface tension of microspheres
l_{cc}	Center to center distance of necked microspheres during sintering
θ	Angle of intersection of the necking area
h_c	Height of spherical cap
A_{hex}	Area of the hexagonal base of the unit cell
V_{unit}	Volume of unit cell
p_s	Scaffold porosity
$V_{unit\ max}$	Volume of unit cell at time $t = 0$
$l_{cc\ max}$	Center to center distance of necked microspheres at time $t = 0$
P_{comp}	The deficient scaffold porosity (or packing density) arising from stacking defects
N_{neck}	The number of necks that a microsphere forms with its neighbouring microspheres
V_{actual}	The actual volume of the unit cell after taking the non-contacting points of the microsphere into consideration
$V_{Microsphere}$	Volume of a microsphere
V_s	Bulk volume of scaffold
V_c	Volume occupied by the interconnected channels

p_g Global porosity of the scaffold

Chapter 1 – INTRODUCTION

1.1 Background

Tissue loss and organ failure resulting from trauma or diseases are major healthcare concerns which affect millions of people worldwide . In the past decade, Tissue Engineering (TE) has become an area of immense research owing to its tremendous potential in tissue/organ repair and replacement therapies. It is an interdisciplinary field that combines principles of engineering and life sciences for the creation of viable, off-the-shelf substitutes that can circumvent the limitations of existing clinical treatments for failing organs and tissues, e.g., the severe shortage of donor organs, the issue of chronic rejection and the risk of donor site morbidity . Initially focusing on skin tissues, an increasing number of other tissue types are now being actively researched as well e.g., liver, heart tissues, cornea, cartilage, bone, blood vessels and esophagus.

The main TE approaches include the injection of cells alone, development of cell-encapsulated systems and the transplantation of cells onto scaffold for tissue growth . Among these strategies, the combination of cells and scaffolds is one of the most widely explored concepts to engineer a tissue . In this approach, the scaffold serves as a biocompatible, supporting framework for cell organization and tissue development in a three-dimensional (3D) environment. The process involves seeding the scaffold with the relevant cells and culturing the cell-seeded scaffold for a period of time prior to implantation. Within the scaffold, transplanted cells can adhere, migrate, proliferate and produce new extracellular matrix (ECM) to form ‘new’ tissue as it slowly degrades. Subsequently, the construct is implanted into the host where it continues to develop *in vivo* and remodel itself into the tissue intended for replacement.

In essence, the scaffold functions to mimic the endogenous ECM of natural tissues. By presenting transplanted cells with appropriate structural and biological cues e.g., porosity, pore size and surface chemistry, the scaffold has the potential of supporting and directing the growth process towards a functional tissue equivalent. This concept of scaffolding in tissue engineering is similar to that applied in architectural buildings,

with the exception of the workers being the cells responsible for tissue development. Different types of buildings may adopt different scaffolding designs. Similarly, the diverse nature of tissues will have different ECM structures and thus, different scaffold designs. Through years of numerous research efforts, a vast base of knowledge pertaining to tissue engineering scaffolds has been established. An essential design requirement is for scaffolds to be highly porous with large surface area to volume ratio so as to maximize space for cell adhesion and migration . The pore size of a scaffold has also been shown to affect the degree of infiltration, proliferation and extracellular matrix deposition *in vitro* . Generally, pores that are too small will tend to impede cell infiltration into the scaffold. On the other hand, excessively large pore sizes will result in void areas having little or poor tissue growth due to limited surface area for cell adhesion. Depending on the type of tissues, the pore size requirement of a scaffold may differ as different cells have preference for different pore sizes . In addition to porosity and pore size, several studies have also reiterated the requirement for scaffolds to have fully interconnected and open pore structure to enable the exchange of nutrients and waste between cells and their environment . These parameters e.g., pore interconnectivity, pore size, porosity define the overall architecture of the scaffold and are known to have profound influence on cell behavior and overall tissue development . Thus, the scaffold plays a fundamental role in the tissue engineering process as its overall design and its eventual properties can profoundly influence the overall success of new tissue formation.

Another challenging aspect of the tissue engineering process, in addition to scaffold design, is to develop suitable fabrication method(s) to produce scaffolds that can fulfill the essential design requirements. In general, there are two broad categories of scaffold fabrication methods: conventional techniques and advanced processing methods. Conventional scaffold fabrication techniques are usually manual processes such as solvent casting, particulate leaching , gas foaming , fibre meshes/fiber bonding , phase separation , emulsion freeze drying , solution casting and freeze drying . The inherent limitations of these techniques are well recognized, e.g., the resulting pore architectures of these scaffolds are highly process driven which often results in inconsistent scaffold architecture . Furthermore, there are risks associated with the use of toxic solvents that may be harmful to the cells if they are not properly and completely removed. However, these techniques are still commonly employed

today for scaffold production because of their low cost preparation and ease of fabrication without the need for specialized equipments.

In view of such limitations, advanced methods such as rapid prototyping (RP) or Additive Manufacturing (AM), are seen to be better viable alternatives for scaffold fabrication . RP techniques are additive processes in which physical parts are constructed in a layer-by-layer fashion based on a computer-generated model. Compared with most conventional scaffold fabrication methods, RP offer users significant control over scaffold architecture in terms of part consistency and design repeatability . The ability to construct scaffolds with intricate architectures in a reproducible manner is one of the key advantages offered by these RP techniques. Thus, the application of RP to produce TE scaffolds has been gaining popularity in the field due to their versatile control over scaffold architecture.

1.2 Motivation

The design requirements of a scaffold may differ depending on the types of tissues being engineered. These requirements can be: a) shape and overall size with dimensions from the milli- to centimeter range, b) size and orientation of pores and channels for tissue in-growth in the order of tens to hundreds of microns, and c) the overall configuration of the scaffold. Macro-size features such as channels are desirable for rapid tissue infiltration while micropores are required for tissue development. Previous studies have reported that channels in the range of 350-400 μm enhance cell infiltration into 3D scaffolds, both *in vitro* and *in vivo* . For large tissues (e.g., liver, muscle tissues), the scaffold may be required to have macro channels for perfusion of culture medium via bioreactor systems . At the micro level, numerous studies have shown that the size of the pores elicits different interactions depending on the cell type . For example, it was reported that vascular smooth muscle cells displayed higher cell proliferation and extracellular matrix synthesis in scaffolds with an average pore size of 107 μm , while endothelial cells show a preference for pore sizes less than 38 μm , forming a connected multicellular lining . The configuration of the scaffold refers to its overall structural framework e.g., homogenous scaffold, layered scaffold or functionally graded scaffold. For example,

bone tissues (e.g., femur) are structurally organized in such a way that bone porosity varies from the outer perimeter to the inner regions (cortical and cancellous regions) and can be regarded as a functionally graded structure. An important requirement for a bone scaffold then, is to replicate this porosity gradient across the bone in order to mimic the regional variation of compressive stiffness in native bones. Other tissues may consist of several distinctly different tissue layers (e.g., blood vessels, esophagus) which are essential to normal tissue function. Thus, they require the use of multilayered scaffolds where each layer represents a different porous structure catered to a specific cell layer of the tissue. In some cases, scaffolds must meet certain geometrical and shape criteria (e.g., bones) to fill the defect area. Therefore, a scaffold can be expected to meet a wide range of macro- and micro-scale requirements due to the diverse nature of tissues. The approach to fabricate the scaffold then, becomes an important consideration for tissue engineers since the overall architecture of scaffolds is largely determined by its fabrication process.

As mentioned previously, conventional scaffold fabrication methods have known limitations. The main disadvantage of these fabrication methods is that the resulting scaffolds are often bulky foam structures with inconsistent pore architectures e.g., non-uniform pore sizes, regions of random porosity and/or random pore interconnectivity. This is due to the difficulty in controlling the process that creates the pore structure of the scaffold. For example, in the solvent casting/freeze drying fabrication approach which is commonly used today, the pore structure of the scaffold is formed from the growth of ice crystals during the freezing process. Sublimation of the ice crystals will ultimately create the pores of the scaffold. However, controlling the formation of ice crystals is difficult and often results in a structure with random pore sizes and inhomogeneous regions of porosity. Another commonly employed fabrication approach is the particulate leaching technique. In this process, particulates are dispersed into a polymer solution. After solidification of the polymer, the particulates are dissolved and the spaces created become the pores of the scaffold. The disadvantage of this process is the random pore interconnectivity that it produces. Depending on the shape of the particulates used and how they are arranged in the polymer solution, pore interconnectivity is not always guaranteed especially in cases where the particulates are not in contact with one another. With such unpredictability

in these processes, they are also unable to produce scaffolds with layered structures or functional gradient.

More advance methods of fabrication such as RP technologies have aided in overcoming some of the limitations faced by conventional methods . Using RP, scaffolds with many complex structures can be created as long as their design can be drawn into computer aided design (CAD) data. Thus, it offers advantages like structural consistency as well as part reproducibility since the scaffold is completely fabricated based on computer generated models. However, they are not without limitations. Although having a higher degree of control over scaffold structure, RP techniques have difficulties in producing micron-sized features due to resolution limits . The current resolution of most RP systems operates on a scale of 100 μm or larger. Thus, RP techniques are limited to very large pore sizes and are unable to produce micro-scale pores. Considering that the length scales of structures that govern several cell-specific functions such as adhesion, proliferation and differentiation are in the order of tens of microns, current RP technologies have severe limitations in producing scaffolds that can sufficiently control these functions . Furthermore, RP was originally conceived to produce engineering prototypes rather than bio-functional structures. The potential biomaterials that can be used directly in currently available RP systems for scaffold fabrication are therefore limited. This is because some RP systems use elevated processing temperatures or toxic binders to fuse the raw materials into a continuous 3-D part and they are not designed for non proprietary raw materials. To expand the range of RP-compatible biomaterials, researchers have explored an indirect scaffold fabrication method . The concept of this method is similar to that of the particulate leaching process except that in this case, a sacrificial mold created from a RP system is used instead of using particulates. In this process, the desired biomaterial solution is cast into the mold representing the negative image of the scaffold design. After mold removal, the design is then transferred into the solidified biomaterial. However, even with the indirect fabrication approach, it is still unable to control the size and distribution of micro-pores due to resolution limits . The solidified biomaterial occupying the mold becomes a random porous structure after the mold removal and drying process, resulting in a structure which is almost similar to that obtained using conventional freeze drying approach (see Figure 1.1).

Figure 1.1: Illustration of Indirect scaffold fabrication using RP technique

Thus, to be able to produce scaffolds that can satisfy the complex macro and micro-scale requirements of tissues, the fabrication process must have a sufficient degree of architectural control. From this point of view, RP techniques are preferred scaffold fabrication methods over conventional processes as it is able to produce scaffolds with macro-channels as well as being able to fabricate them in different configurations (layered structures or functional gradient) in a consistent manner. However, in order for RP fabricated scaffolds to control cell-specific functions and behavior, it must also be able to fulfill the micro-scale requirements of tissues. There is thus a need to overcome the resolution limit of RP fabrication so as to address the controllability of scaffold architecture at both micro- and macro-scale levels. This research focuses on developing an RP fabrication method that is able to create scaffolds that can meet the design requirements of tissues at all length scales. As mentioned previously, the indirect RP fabrication method allows a wider range of biomaterials to be used as compared to direct RP fabrication. In the case of direct RP fabrication, it is difficult to

alleviate the problem of resolution constraint unless machine hardware e.g., printhead nozzles, laser source etc, are upgraded. However, this requires back and forth system calibration and testing which may be very tedious, costly, time consuming and may not be technically feasible. The indirect approach, on the other hand, offers us the flexibility to manipulate and/or modify the mold, which forms the basis of the solution adopted in this research to overcome the resolution limit. This approach is different from indirect fabrication methods previously adopted by researchers whose aim is to widen the scope of RP-compatible biomaterials for scaffold fabrication but offers no control over micro-scale features. The concept behind our strategy is a totally inverse fabrication approach in which the entire architecture of the scaffold e.g., pore size, pore interconnectivity, geometry, shape, porosity etc. is shaped using the lost-mold technique. As such, the mold would represent the negative replica of the entire scaffold architecture. For the mold to achieve this function, it must consist of features to represent the different aspects of the scaffold e.g., channels, pores etc. This can be achieved by modifying the RP mold into a composition that is exactly the negative image of the desired scaffold structure.

In this research, we fabricate this modified mold by combining a RP mold with filler materials. These ‘filler’ materials are meant to fill up the spaces of the RP mold and they represent the micro-pores of the scaffold structure while the RP mold itself represents the macro-channels of the scaffold. The final scaffold can be obtained by simply casting the scaffold material into the mold and leaching off the mold completely. In a sense, this process becomes equivalent to the particulate leaching process. In this manner, scaffolds with precise and customizable micro-pores and configurable macro-features e.g., channels can be fabricated by controlling the size of the filler materials as well as the design of the RP mold.

Components commonly used in particulate leaching processes are suitable candidates as the filler material. However, these components e.g., salt crystals are irregular in shape and are known to form scaffolds with random pore interconnectivity. To have rigorous control over pore size and to ensure interconnectivity between pores, the particulates have to be regular in shape and size so as to allow maximum packing. This is because interconnectivity is maximized when particulates are arranged in the closest packed arrangement. For this research, monodispersed microspheres will be

used as the filler material to control micro-scale features of the scaffold e.g., pore size and interconnectivity owing to their consistent, spherical shape, size and their tendency to maximize packing. To incorporate channel structures within the scaffold, the selected RP system must be able to produce parts with zero porosity. Porous parts will allow the scaffold material to infiltrate during the casting process and the resulting channels will not be clear channels but with traces of scaffold material upon removal of the part. Therefore, powder based RP systems e.g., Selective Laser Sintering (SLS) will not be suitable for this purpose as they tend to produce porous parts. Instead, a liquid based RP system, more specifically the Objet Eden 350V printer was selected in this work. This RP system operates on the PolyJet™ technology which creates 3D models using a layer-by-layer photopolymer curing process. The ability to fabricate non-porous parts and the high resolution achievable by the 3D printer are the primary reasons for this selection. Eventually, the RP mold and microspheres are fused together to represent the desired structure of the entire scaffold.

As with most research work, the development of this proposed inverse scaffold fabrication method is iterative. The three main stages of work in this research are 1) understanding the capabilities and limits of the Objet 3D printer, 2) identifying the important process parameters of the inverse scaffold fabrication method that controls the scaffold parameters such as pore size, porosity and pore interconnectivity, and 3) developing the entire scaffold fabrication methodology. To better understand the fundamentals of the RP process, a simple analytical model will be established to correlate the operating parameters of the RP system to the dimensions of the fabricated part. In addition, the important parameters of the particulate leaching process that controls the pore structure of the scaffold e.g., microsphere size, sintering time and temperature etc., will be analyzed and analytical models established to correlate these parameters. Finally, the fabricated scaffolds will be assessed for toxicity and their feasibility for TE applications.

1.3 Objectives

Based on the motivations of this project, the objectives are:

- (i) To develop a novel scaffold fabrication approach based on the concepts of AM fabrication and the particulate leaching method that will allow sufficient control over both micro and macro-scale features of the scaffold.
- (ii) To alleviate the issue of resolution limitation that has been long associated with the use of additive manufacturing techniques in TE scaffold fabrication.
- (iii) To create and illustrate an inverse concept of scaffold fabrication in which the entire pore structure of the scaffold is shaped via a single mold using a lost mold technique.

1.4 Scope

The scope of the project includes the following:

- (i) Study the operating process of the Objet 3D printing system and understand the differences in the two printing modes offered by the Objet printer.
- (ii) Explore the production of polystyrene microspheres e.g., fabrication, separation and storage, and also to explore methods to arrange the microspheres into a close packed arrangement.
- (iii) Establish a mathematical model that correlates microsphere sintering time to scaffold architectural parameters e.g., pore interconnectivity and porosity.
- (iv) Design and fabricate a sacrificial mold by fusing polystyrene microspheres with parts built from the selected RP system. This sacrificial mold will represent the negative replica of the entire scaffold architecture.
- (v) To demonstrate the feasibility of the proposed inverse fabrication approach by producing scaffolds having an interconnected, 3D network of channels embedded within a highly porous structure with consistent and customizable pore sizes.

(vi) Conduct cytotoxicity assessment on the fabricated scaffold to address the concern of possible toxic leachables derived from the proposed inverse fabrication technique.

1.5 Organization of Report

The report is organized as follows:

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

Chapter 2 consists of literature reviews that cover the main aspects of this research, namely, tissue engineering, scaffolds and scaffold fabrication processes. Biomaterials that have been used in TE are also described. The limitations of the current scaffold fabrication techniques are identified and discussed.

Chapter 3 discusses the operating process of the liquid based RP system and theory underlying the PolyJet™ printing technology. Investigative work related to dimensional accuracy is presented as well.

Chapter 4 discusses the fabrication process, handling and storage of the polystyrene microsphere with some experimental work. Methods to align the microspheres into a hexagonal closed pack (HCP) arrangement are also explored.

In Chapter 5, a theoretical analysis that correlates the sintering time interval to the scaffold porosity and pore interconnection size is presented.

The methodology undertaken in this research is presented in Chapter 6. Here, the entire process and protocol of the proposed inverse scaffold fabrication method are outlined. The steps and procedures related to both mold and scaffold fabrication are described. To verify the analytical model, experimental procedures pertaining to

characterization of the fabricated scaffolds are presented. Experimental procedures involving preliminary *in vitro* work are presented as well.

In Chapter 7, the results of the experiments are presented. Subsequent discussion focuses on the characterization of the scaffolds, the verification of the analytical model as well as *in vitro* analysis of the cells on these fabricated scaffolds.

A conclusion arising from the work done is presented in Chapter 8. Finally, future work continuing from the status of the present research is also described in this chapter.

Chapter 2 – LITERATURE REVIEW

A prevalent approach in tissue engineering is the culturing of donor cells that are seeded onto porous 3D scaffolds, and subsequent implantation of the cell/scaffold construct into the anatomic location. This approach involves firstly, the formation of a mass of tissue by culturing the cell/scaffold construct *in vitro* in which the cells proliferate and produce new extracellular matrix (ECM) components. Secondly, the construct is then implanted into the appropriate anatomic location where *in vivo* remodeling is intended to restore the normal tissue structure and function (see Figure 2.1).

Figure 2.2: Scaffold-guided tissue regeneration

The scaffold-guided tissue regeneration strategy provides an actual physical 3D structure of the organ/tissue to be regenerated to which cells can attach on. The goal is to allow the cells to replicate and/or differentiate while being attached, and eventually organize into normal functional tissue as the scaffold slowly degrades. Growth factors are often incorporated within the scaffold to enhance the proliferation of cells and to drive their differentiation from a non specific state towards a desired lineage that exhibits the functions of the target tissue.

Eventually, the scaffold will disintegrate and be excreted by the body through natural metabolic means, leaving behind the newly regenerated tissue. This approach has been extensively researched to engineer various tissue analogs including skin , cartilage , bone , liver and blood vessels .

2.1 Essential Properties of TE Scaffolds

The tissue scaffold serves as a temporary template for cell attachment and organization of tissue growth in a 3D environment. In fact, a good scaffold should function in much the same way as the body's own ECM, allowing normal cellular and physiological processes to occur. The past decade of tissue engineering research which has focused on almost all known tissues e.g., cartilage, bone, liver, intestines, esophagus, skin etc., has established a vast base of knowledge pertaining to tissue engineering scaffolds. The following has been identified as essential scaffold properties that are crucial for proper cell growth :

2.1.1 Scaffold Porosity

Cells, following initial attachment, will migrate into the scaffold, proliferate and differentiate to express their normal phenotypic functions. For example, in some cases such as fibroblasts, the cells synthesize new ECM as part of the regeneration process. In this regard, scaffolds must be highly porous (see Figure 2.2) with large surface area to volume ratio so as to maximize space for cell adhesion and migration . Freed *et al* proposed that scaffolds should possess a porosity of at least 90% to allow for adequate nutrient diffusion and tissue ingrowth during *in vitro* culture. Bose and Bignon showed that the increase in scaffold porosity is often accompanied by an increase in cell proliferation. They suggested that the increased proliferation in scaffolds with higher porosity volume is primarily due to the effect of higher surface area per unit volume. However, the mechanical strength of the scaffold structure is compromised for the lost of volume to porosity. Hence, the design process of a scaffold often needs to resolve these conflicting design requirements.

Figure 2.3: Porous structure of PLLA scaffold

2.1.2 Optimal Pore Size

In addition to scaffold porosity, the size of pores will also determine the ease at which cells can migrate into the scaffold. A densely packed pore structure with small pore sizes generally suggest a high surface area to volume ratio, and hence a high porosity. However, pores that are too small will tend to impede cell infiltration into the scaffold. This is also dependent upon the type of cells being used as studies have shown that different cell types display preferences to different pore sizes. For example, in regenerating bone tissues, some researchers have indicated the need for pore sizes ranging from 200 to 400 μm for optimal cell growth . Zetlinger *et al* also reported that microvascular epithelial cells grew well on scaffolds with pore sizes less than 38 μm whereas vascular smooth muscle cells preferred pore sizes greater than 63 μm . Dermal fibroblasts, on the other hand, showed no dependency on pore size. When pores employed are too small, pore occlusion will occur and eventually prevent further cell penetration into the scaffold .

While the pore sizes of scaffolds have shown to affect not only the degree of cell infiltration and proliferation *in vitro* , it has also been reported to affect *in vivo* responses such as fibrosis and angiogenesis . The propensity of implanted materials to shift from avascular, fibrotic healing to angiogenesis in response to varying pore sizes has been demonstrated in several studies . These studies have shown that to minimize fibrosis and to establish blood supply within the newly-grown tissue, pore sizes of the

scaffold should be in the range of 20 to 60 μm . This angiogenic response is crucial to the viability of the newly regenerating tissue and its integration with the host tissue following implantation. Furthermore, it has become clear from several studies that these parameters are highly potent regulators of stem cell differentiation

Depending on the tissues being engineered, the scaffold may also have to consist of different pore sizes. For example, the esophagus is a multilayered tissue consisting of the epithelial layer and the muscle layer (see cross sectional view of the esophagus in Figure 2.3). Therefore it is imperative for the scaffold to be customized in a way that it possesses a range of pore sizes to mimic its native structure and functions. In the case of the esophagus, the pore sizes in the luminal layer of the scaffold must be small to encourage epithelialization. Zeltinger *et al* proposed that scaffolds with pores of less than 38 μm be used to grow the epithelial lining since larger pores will

Figure 2.4: Cross-sectional organization of tissue in the human esophagus: Mucosa (M) consisting of epithelium (E) and the muscularis mucosa (MM), the submucosa (SM) and muscularis externa showing the inner circular (IC) and outer longitudinal (OL) muscle layer

result in discontinuous pockets of epithelial cells. As for the muscle layer, the pores should be large and well interconnected to the interior to facilitate cellular infiltration, ECM deposition and vascularization in the interior regions of the scaffold. Here, pore sizes of more than 68 μm are recommended for high seeding efficiency and effective exchange of nutrients and waste *in vitro* .

2.1.3 Pore Interconnectivity

While scaffolds require high porosity with appropriate pore sizes, the interconnectivity between individual pores is also another important consideration. The interconnection serves as a link or pathway between pores for diffusion of nutritional elements and extracellular fluid circulation as well as cell movement from one region to the other. Thus, a highly porous microstructure with interconnected porous networks is critical in ensuring uniform cell distribution, cell survival, proliferation and migration *in vitro*. Several studies have reiterated the need for scaffolds to have a fully interconnected and open pore geometry to enable the exchange of nutrients and metabolic waste between cells and their environment . This is especially important in growing the large tissues since high rates of mass transfer can be expected owing to the high metabolic demands of its cells. In the case of closed pore structures where individual pores are not interconnected, nutrient and waste transport will be inefficient even in a highly porous matrix. Issues related to cell migration and tissue ingrowth will be implicated as well.

A scaffold with a highly interconnected pore structure is essential for a number of reasons. First, local disturbances in culture environment e.g., changes in osmotic pressure and pH manifested through the accumulation of metabolic waste derived from cells and/or acidic scaffold degradation by-products are detrimental to cell growth . This is more pronounced in later stages of tissue growth when the developed tissue becomes denser and thus reducing the diffusion rate for waste removal and nutrient inflow. Secondly, nutrients will be unable to reach the cells residing in the scaffold interior once the cells at the surface start growing in multiple layers. Therefore, it is extremely crucial for scaffolds designed for large tissue culture to be

exposed at all times to sufficient quantities of neutral culture media, and hence the need for a highly interconnected porous network.

In an experiment conducted by Lu *et al* , cells were observed to be spreading across the interconnection between pores while migrating into the scaffold. The study also showed that an interconnection size of 50 μm is needed to favor bone growth inside the scaffolds. He argued that porosity and pore interconnection density are more important than pore size. In recent development, researchers have been exploring the use of scaffolds with interconnected channels so as to improve the interconnectivity within the scaffold and also to simulate the vascular bed in natural tissues . Interconnected channel networks with micron sized diameters can be achieved using RP techniques. However, the range of channel diameter that can be produced is ultimately dependent on the build resolution of the RP system. The resolution in turn, is dependent on the nature of the RP process.

2.1.4 Scaffold Degradation

As the eventual goal is the creation of a completely new biological tissue, the scaffold must eventually disappear and be replaced by newly synthesized ECM components and cells. The process of scaffold degradation can take place both *in vitro* and *in vivo*. More importantly, the degradation by-products must not elicit any cytotoxic or adverse responses (severe inflammation) to the surrounding host tissue. Some polymer-based scaffolds e.g., PLA, PGA etc produce degradation by-products which are known to potentially create locally toxic environments that are inconducive to cell growth, thus impeding proper tissue regeneration . In this case, a highly interconnected pore structure e.g., scaffold with interconnected channel networks, is necessary so as to facilitate the diffusion of degradation products out of the scaffold to be expelled via normal metabolic pathways.

Ideally, the rate of scaffold degradation should also be consistent with the rate of tissue regeneration . A fast degradation rate, and hence, the loss of mechanical stability in the early phases of tissue development may be detrimental to proper regeneration as was demonstrated in the comparative study by Jansen *et al* . In his

work, anastomotic leakages and stenosis were evident due to early degradation of PGA mesh whereas the use of non-biodegradable Poly(vinylidene fluoride) (PVDF) mesh resulted in a leakage-free, non-stenotic construct. Of course, the choice of degradable scaffold material could be an important factor in the outcome of the regenerative process. Thus, both the scaffold material and its rate of degradation (dependent on the intrinsic properties and also the design of the scaffold) are factors that must be considered carefully in the tissue engineering process.

For polymer scaffolds, there are two types of scaffolds biodegradation . First, the scaffold can be degraded by simple chemical hydrolysis of the backbone of the polymer. This can occur *in vitro* when the scaffold is in contact with the culture fluid, and also *in vivo* when the scaffold is in contact with body fluids. Water penetrates the bulk of the device, attacking the chemical bonds and converting long polymer chains into shorter fragments. This occurs in the amorphous phase and initially there is a reduction in molecular weight without a loss in physical properties. The reduction in molecular weight is followed by a reduction in physical properties, as water begins to cause fragmentation to the device. Fragments that have a molecular weight below 50,000 are filtered and excreted by the kidney without being fully metabolised. Next, enzymatic attack and metabolization of the fragments occurs, resulting in a rapid loss of polymer mass. This type of degradation, where the rate at which water penetrates the device exceeds that at which the polymer is converted into water-soluble materials, is called bulk degradation. This degradation occurs throughout the entire scaffold structure.

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

The degradation mechanism is the result of many interrelated factors that include hydrophilicity of the polymer backbone, number of reactive hydrolytic groups in the backbone, degree of crystallinity, presence of catalysts, additives, impurities, or

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

2.1.5 Surface Properties

Research findings from *in vitro* tissue engineering studies employing cellularised scaffolds have been very encouraging . Consequently, scaffolds should be aimed at improving seeding efficiency and cellular retention if tissues are to be engineered *in vitro* before implantation. To facilitate these processes, the scaffold must first provide a biocompatible surface that promotes cell recognition and adhesion. The physical properties and physiochemistry of the scaffold's surface are important factors that influence cell attachment, migration and intracellular signaling *in vitro* and cell recruitment and healing at the tissue-scaffold interface *in vivo* . Surface roughness and topography are examples of such physical parameters which has been strongly associated with cell-scaffold adhesion. Also, it was also shown that the chemical composition of the scaffold's surface plays a decisive role in cell adhesion and further behavior of cells after contact. This adhesion is mediated through specific integrin receptors (see Figure 2.4) that are present on cell membranes . The attachment of these receptors to specific ligands on the scaffold surface is crucial for further migratory, proliferation and differentiation behavior of anchorage dependent cells. As such, current strategies to control the proliferation and other behaviors of cells on scaffold are by patterning the scaffold surfaces with biomolecules containing these ligands .

Biocompatible surfaces can be achieved by generating hydrophilic surfaces or topographical features that can interact effectively with cells. For example, esophageal epithelial cells have been demonstrated to display better attachment on electrospun poly(L-lactide-co-caprolactone) (PLLC) scaffolds when nano-size pores are introduced onto the surface of each individual fiber as compared to non-porous electrospun controls . Bioactive surface modification or immobilization with ECM proteins has also been reported to improve cell adhesion. By exploiting the natural

cell affinity of these proteins, cell-scaffold interaction can be enhanced through integrin-mediated adhesion. This was illustrated experimentally by Zhu *et al*, where immobilized fibronectin and collagen on scaffolds were effective in supporting the attachment of all esophageal cell types . Furthermore, studies have also shown that cellular functions involving proliferation and ECM production can be affected by changes in surface chemistry and topology . As tissue engineering research moves towards the use of serum and animal protein-free culture conditions due to associated health risk and regulatory hurdles concerning FDA approval, the ability to tailor the surface properties of scaffolds will be essential in modulating cell attachment.

Figure 2.5: Cell integrin-ligand mediated adhesion

2.1.6 Mechanical Strength

The mechanical strength of most tissues is associated with its primary functions. For example in the case of the esophagus, its mechanical properties are vital for its peristalsis function since it is frequently subjected to varying stresses and strains caused by the passage of food bolus. An extensive mechanical study on porcine esophagus have shown that it is a highly elastic, tubular tissue exhibiting anisotropic behavior; it is stronger longitudinally than radially, but the opposite is observed for extensibility . Vanags *et al* reported the ultimate mean strength (circumferentially and axially) of a normal human esophagus to be at 1.41 MPa and 2.19 MPa, with mean elastic modulus of 1.44 MPa and 2.30 MPa respectively. Similarly, the esophageal scaffold designed for *in vivo* implantation, or for *in vitro* tissue development should possess sufficient elasticity in the radial direction for it to expand and relax easily for

smooth ingestion of food. In the axial direction however, a lower extensibility is favored so as to prevent accumulation of high stresses at the connective tissue interface between the esophageal scaffold and the surrounding tissues when under tension .

In the same manner with other tissue types, scaffolds should have mechanical properties e.g., elasticity, yield strength etc. that match or are close to that of the desired native tissue. The mechanical strength of the scaffolds must also be sufficient in order to maintain the 3D porous structure required for cell in-growth and nutrient transport during *in vitro* culturing or after *in vivo* implantation . Upon implantation, the degradable scaffold should retain sufficient mechanical strength to withstand any *in vivo* stresses and physiological loadings imposed on the engineered construct .

Although an important aspect of scaffold design, the biomechanical properties of the scaffold cannot be over-emphasized as the long term success depends on the engineered tissue *in vivo*. Since the scaffold inevitably undergoes the process of degradation, the mechanical properties of the evolving cell/scaffold construct *in vivo* will be different from that before implantation. This is further complicated by the complex *in vivo* environment in which the degradation of the implant can be affected by several factors e.g., location of implant, health of the host, pore architecture of the scaffold, enzymatic and local cellular activity . Therefore, it is difficult to predict the eventual mechanical strength of the scaffold and also if it will remain compliant after a long period of stay *in vivo*.

2.2 Scaffold Materials

The properties of the scaffold material will determine several important aspects of the scaffold such as mechanical strength, degradation rate as well as surface chemistry which can influence the outcome of the whole regeneration process. Over the past decade, a variety of materials have been evaluated as possible scaffold material for tissue engineering applications ranging from synthetic polymers e.g., PGA , Poly (L-lactide-*co*- ϵ -caprolactone) (PLLC) , Polylactic acid (PLA) poly(lactide-*co*-glycolic

acid) (PLGA) to biologically derived materials e.g., collagen and decellularized matrices.

2.2.1 Biological Materials

These are naturally derived materials which can be used as a scaffold. A good example would be collagen which is the major protein component of mammalian connective tissue, accounting for 30% of all protein in the human body . It is found in every major tissue type which requires strength and flexibility. Clinical applications include the manufacture of sutures, haemostatic agents (powder, sponge, fleece), blood vessels by extrusion tube, tendons and ligaments, dermal regeneration for burn treatment and peripheral nerve regeneration .

Biological materials have generally showed promising results. This can be attributed to the presence of cell integrin receptor domains which are abundant in these natural materials . The work of Beckstead *et al* who studied the behavior epithelial cells on a variety of scaffolds is a good example illustrating the superiority of biological materials. Cells grown on Alloderm™, a commercially available decellularized matrix, were observed to have better morphology displaying stratified cell layers with differentiation states similar to that of a normal epithelial lining as compared to those grown on synthetic scaffolds. These domains such as Arginine-Glycine-Aspartic (RGD) tripeptide sequences mediates cell adhesion and are also involved in a diverse family of intracellular signaling pathways which regulate several cellular functions (e.g., migration, differentiation, proliferation) . Decellularized matrices are especially rich in these cell-recognition sites.

2.2.2 Synthetic Materials

Although the eventual goal is to provide a scaffold that will eventually be degraded and replaced by proliferating cells and newly synthesized ECM, it is important to bear in mind that they must have appropriate degradation rates such that a sufficient amount of tissue can be formed before the onset of bulk scaffold degradation in which the entire mechanical integrity of the scaffold is lost. This is where synthetic polymers

become an attractive class of material. A key advantage in using them is that they can be optimized for their mechanical properties and degradation rate depending on their applications . These properties are critical issues concerning the long term success of a scaffold and such flexibility is mostly absent in naturally derived materials. Although lacking in cell recognition sites, these scaffolds can be grafted with ECM proteins via surface modification techniques to promote cell attachment. In order to harness the advantages associated with both synthetic and biological polymers, future research must be directed towards surface modification and functionalisation approaches. In this way, essential biological cues can be imbued onto mechanically and functionally compliant materials for tissue engineering applications.

Among the different materials used for scaffold fabrication, polymers have been extensively explored in the area of tissue engineering. On the other hand, naturally derived materials are more advantageous because of their inherent properties of biological recognition, including presentation of receptor-binding ligands and susceptibility to cell-triggered proteolytic degradation and remodeling . Gelatin, a natural biopolymer derived from collagen by controlled hydrolysis was selected in this work based on its excellent biocompatibility and lack of antigenicity . In addition to its biological origin, it is commercially available at relatively low cost, and thus being widely used in the pharmaceutical and medical fields. Furthermore, it will not decrease the local pH during degradation as compared to synthetic polymers e.g., PLA and PGA that produce fragments of acidic products. Additionally, gelatin scaffolds have been observed to promote angiogenesis which is essential for the survival of engineered tissues. . It is suitable for use in both hard and soft tissue regeneration .

2.3 Scaffold Fabrication Methods

To date, there has been extensive research work dealing with various fabrication methods to produce TE scaffolds. The most commonly used fabrication methods are conventional methods like solvent casting/particulate leaching , gas foaming , fiber bonding , phase separation , freeze drying , and membrane/sheet lamination . More advanced methods of scaffold fabrication include RP techniques and electrospinning.

In the following sections, the working principles, advantages and disadvantages of these fabrication methods will be discussed.

2.3.1 Conventional Fabrication Methods

Conventional methods of scaffold fabrication have been widely reviewed. In general, these methods are easy to apply and do not require the use of specialized equipment.

2.3.1.1 Gas Foaming

The gas foaming technique can be used to produce highly porous polymer scaffolds without the use of organic solvents. In the fabrication process, Carbon dioxide (CO₂) is often used as a ‘blowing agent’ in its gaseous or supercritical form. When exposed to high pressure CO₂, the polymer matrix gets saturated with the gas. The solubility of the gas within the polymer is then rapidly decreased by lowering the pressure to atmospheric levels. This decrease in pressure creates a thermodynamic instability for the dissolved CO₂ resulting in the nucleation and growth of gas bubbles in the interior of the polymer matrix. Thus, the CO₂ behaves as ‘porogens’ that form the scaffold pores in the fabrication process. Salerno *et al* created PCL scaffolds with multi-scaled porous networks using the gas foaming method (see Figure 2.5). Mooney *et al* successfully produced PLGA scaffolds with an average pore size of about 100 µm and porosity of up to 93%. However, it was reported that this method resulted in the presence of a relatively non-porous skin layer on the scaffold surface due to rapid diffusion of the dissolved CO₂ from the surface. Although a relatively simple process that can produce highly porous scaffolds, it tends to create closed pore structures with limited pore interconnectivity. Attempts were made to minimize these drawbacks by combining the gas foaming process with the particulate leaching technique. For example, Harris *et al* fabricated PLGA scaffolds by subjecting PLGA and salt particles to the gas foaming process. The polymer and salt particles fused to form a continuous matrix with the salt particles subsequently leached out of the scaffold using water, leaving behind a highly porous foam with good pore interconnectivity.

From a tissue engineering perspective, the main advantage of this fabrication method is the ability to fabricate polymer scaffolds without the need to use solvents as it is not required to leach the CO₂ from the scaffold unlike the porogens used in particulate leaching. This may be beneficial for tissue engineers incorporating bioactive molecules e.g., growth factors, proteins within the scaffold as they can be denatured when in contact with the solvents. Similar to the particulate leaching process however, it is not possible to fabricate scaffolds with complex architectures using this method. The size and distribution of pores are also random and difficult to control.

Figure 2.6: PCL scaffolds fabricated via gas foaming

2.3.1.2 Solvent Casting/Particulate Leaching

Particulate leaching is one of the earliest and most commonly adopted methods for scaffold fabrication. This method, first described by Mikos *et al* , involves the dispersion of micron sized particles in a polymer solution. Upon solidification of the polymer, the particles are subsequently removed from the matrix and the spaces created upon their removal become the internal pores of the entire polymer structure (see Figure 2.6). In essence, these particles, which are more commonly addressed as porogens, are important elements of the fabrication process as they ultimately shape the entire pore structure of the scaffold. Examples of commonly used porogens include minerals such as salt crystals, sugar particles, gelatin particles and organic materials such as wax. Scaffolds fabricated using this method usually possess a high porosity. In the work of Murphy *et al* , sodium chloride (NaCl) crystals were used as porogens to create highly porous PLGA scaffolds. In a separate study by Shastri *et al* ,

the use of wax porogens was reported to be capable of fabricating PLLA scaffolds of up to 87% porosity.

Figure 2.7: Fabrication of scaffold using the porogen leaching method

Altering the porogen/polymer ratio allows adequate control over the porosity of the resulting scaffold while the size and shape of pores within the scaffold can be defined by changing the size and the type of the porogens being used. However, this method still suffers from some disadvantages:

- 1) **Limited to simple structures:** The thickness of the scaffold that can be fabricated is limited due to the difficulty in leaching out the porogens deep within the scaffold interior. It is also not possible to produce scaffolds with complex architectures or macro-features e.g., channel networks within the scaffold using this method. Additional processes and/or equipment may have to be involved to fabricate complex structures. For example, Widmer *et al* used solvent casting followed by extrusion, to form tubular shaped scaffolds before leaching out the salt porogens to generate PLGA and PLA scaffolds with a pore size of 5 - 30 μm and porosity in the 60%–90% range.
- 2) **Toxic solvents:** Depending on the type of porogen used, toxic solvents may have to be used to dissolve them. For example, hexane was used to leach out wax porogens in the work of Ma *et al* . This may induce potential toxic residuals within the scaffold which may cause adverse reactions to cells.
- 3) **Limited control over internal architecture** (see Figure 2.7): The natural porogen dispersion within the polymer solution allows very little control over the internal scaffold architecture e.g., distribution of pores and pore interconnectivity etc.

As such, the interconnectivity between pores is not always guaranteed. This is due to the limited contacts between the porogens during the process of polymer solidification. Subsequent leaching of porogens will result in a structure comprising of a mixture of both interconnected pores as well as closed pores (pores that do not connect to neighboring ones).

Figure 2.8: Scaffolds illustrating pores with limited interconnection to other pores as well as closed pores

2.3.1.3 Emulsion/Freeze Drying

This fabrication process involves the creation of an emulsion by homogenization of 2 solvents that are immiscible in one another. First, the polymer is dissolved in a suitable solvent (e.g., PLA in dichloromethane) and a second immiscible solvent (which is usually water) is added to the polymer solution. The mixture is then thoroughly mixed to homogenize the two liquid phases, thus forming an emulsion. Before the two liquid phases can separate, the emulsion is rapidly cooled (e.g., by

means of pouring into a mold and immersion into liquid nitrogen) to solidify the emulsion. The frozen emulsion is subsequently freeze dried to remove the solvent and water, thus creating a solidified, porous polymeric foam structure. The formation of pores in this fabrication process arises from the frozen liquid crystals. After the freeze drying step, the spaces created upon the sublimation of the frozen crystals become the pores of the scaffold (see Figure 2.8). Whang *et al* created PLGA scaffolds using the emulsion-freeze drying method and investigated the effect of average pore

Figure 2.9: Illustration of the emulsion/freeze drying approach

size and protein loading on protein release kinetics. In a separate study by Moshfeghian *et al*, the microarchitecture of emulsion-freeze dried chitosan-PLGA scaffolds was reported to be influenced by the freezing rate and temperature. Furthermore, by controlling the concentration of chitosan, scaffolds with a porosity

exceeding 90% can be fabricated. Sultana *et al* incorporated HA nanoparticles in poly(hydroxybutyrate-co-valerate) (PHBV) polymer to fabricate osteoconductive composite scaffolds. PHBV and HA/ PHBV scaffolds were made using the emulsion/freeze-drying technique. It was observed that the scaffolds were highly porous with pore size ranging from several microns to around 300 μm .

Although a relatively simple method without the need for specialized equipment, it is difficult to control the size of pores and porosity is often irregular. It has also been reported to create closed pore structures. Compared to the particulate leaching process, this method allows a faster fabrication since it does not require a leaching step, although it still requires the use of solvents.

2.3.1.4 Thermally-Induced Phase Separation

Similar to the emulsion/freeze drying technique, the phase separation procedure requires the use of a solvent with low melting point which is easy to sublime. This process is based on the thermodynamic and kinetic behaviour of the polymer solution with the variation of the solution temperature. When the solvent temperature decreases, the crystallization of the solvent can induce phase separation of the polymer solution, a process defined as solid–liquid phase separation (see Figure 2.9). The crystallization process forces/expels the polymer from the solvent crystallization front . Subsequent freeze drying causes removal of the solidified solvent phase, resulting in a highly porous polymeric scaffold. The space originally occupied by the crystallized solvent would become the pores in the scaffolds. The pore morphology of the porous scaffold can vary depending on the polymer, solvent, concentration of the polymer solution and the phase separation temperature.

Figure 2.10: Illustration showing the solvent phase and polymer phase after crystallization process

The phase separation method has been used to produce porous scaffolds covering a wide range of polymers including PLA, PDLA and PLGA. Furthermore, composite scaffolds like amorphous calcium phosphate/PLA and PLA/Bioglass can be fabricated. Gong *et al* fabricated porous PLLA scaffolds via phase separation which was subsequently filled with agar hydrogel containing chondrocytes as a cartilage implant. A separate study by Rowland *et al* showed how polyurethane (PU)/PLGA scaffolds can be fabricated using this method. Compared with scaffolds fabricated from PLGA or PU alone, the composite PU/PLGA scaffold displayed better morphological, mechanical and cell adhesion and growth supporting properties.

In this fabrication method, the control over scaffold pore structure is limited as it is difficult to control the rate and direction of crystal growth as well as their distribution. Another very common drawback of this fabrication method is the occurrence of surface skin covering the scaffold after the freeze drying step. During freeze-drying, the polymer matrix is unable to resist the interfacial tension caused by evaporation of solvent. Thus, the porous structure collapses and dense skin layers occur in the resulting scaffolds, which may hinder cell infiltration into the scaffold interior.

2.3.1.5 Reverse Templating

Similar to the solvent casting/particulate leaching method, the reverse templating technique uses leachable porogens but requires an additional step. This step fuses the porogens together (by means of sintering) into a template before casting of the polymer solution (see Figure 2.10). The sintering process induces fusion between the porogens and fixes/secures the whole structure in place. In this manner, the natural dispersion of porogens in the polymer solution is eliminated and contacts between porogens are improved. Upon casting and solidification of the polymer solution, the template is then leached out of the scaffold matrix, resulting in an interconnected porous structure.

Figure 2.11: Illustration of the reverse templating process

A few research groups have attempted to fabricate scaffolds using the reverse templating technique by using templates created from microspheres. In a study by Liu *et al*, paraffin wax microspheres were poured into a Teflon mold and sintered at 37 °C to fuse the wax particles into a template. The resulting gelatin scaffolds created from these templates displayed an interconnected structure with spherical pores. Choi

et al fabricated chitosan scaffolds using templates created by sintering PCL microspheres at 45°C. As the microspheres were assembled using an orbital shaker before the sintering process, the resulting template consists of orderly aligned microspheres. Thus, the pore structure of the chitosan scaffolds resembles that of an inverse opal. Uchida *et al* employed a different approach to assemble the microspheres into a template. Using sugar microspheres that were encapsulated with ferrite particles, the magnetized microspheres were attracted by a magnet to form a self assembled template which were subsequently used to fabricate highly porous PLLC scaffolds having an average pore diameter of 160 μm .

2.3.2 Rapid Prototyping Techniques

Rapid prototyping (RP), or Additive Manufacturing (AM) as it is now endorsed by ASTM, encompasses a group of techniques that allow highly complex, 3D models to be fabricated directly from computer aided design (CAD) data. They are additive processes in which parts are constructed in a layer-by-layer fashion and have been adapted as a means of scaffold fabrication . The process in general, comprises the CAD design of the scaffold whereby the data of the model is transferred to the RP system expressed as a series of individual cross sections. The RP system, guided by a computer program, then fabricates the scaffold layer by layer according to the cross-sectional data until the built is complete. The more prominent RP technologies for scaffold fabrication include selective-laser sintering (SLS) , three dimensional printing (3DP) and fused deposition modeling (FDM) . As RP was not originally intended for bio-engineering applications, researchers often had to customize the RP technique to be tissue-engineering specific by one of three approaches, namely designing new biomaterial that fits the processing window of the commercial RP systems, modifying the commercial system to tolerate non-proprietary biomaterials or developing new RP systems that use biomaterials directly . The use of RP techniques for scaffold fabrication can be grouped into two broad categories: direct and indirect RP techniques.

Direct RP process: In direct RP fabrication, the scaffold is directly fabricated on the building platform using RP systems. Based on the model assembly approach, this

technique is further categorized into melt deposition techniques and particle bonding techniques.

- In melt deposition techniques, the material is extruded through an orifice while the orifice is moving across the X-Y plane, forming a single layer. The material layer then cools, solidifying itself and to the previous layer. Representative systems using melt deposition are FDM and 3D Bioplotting.
- In particle bonding techniques, the particles are selectively bonded either by laser, which sinters the particles together, or by some kind of binders which fuses the particles. Typical systems using such techniques are SLS and 3DP respectively.

Indirect RP process: Instead of fabricating the scaffold directly, the indirect RP approach creates a master pattern of the scaffold and then use it to produce a sacrificial mold. The mold is then cast with the scaffold material and subsequently removed to produce the final scaffold. This multi-step fabrication approach allows the tissue engineer to control the internal architectural configuration of the scaffold. In addition, depending on the type of raw materials used to fabricate the sacrificial mold, indirect RP methods can allow the use of a wider range of scaffold materials, making it possible to use composite blends that may require conflicting processing demands. Common RP systems employed for scaffold fabrication using the indirect techniques are Model Maker II (MMII) and SLA

2.3.2.1 Fused Deposition Modeling (FDM)

FDM is a extrusion based RP system (schematic diagram of the process shown in Figure 2.11), which uses material filaments as raw building materials. The filament is fed into an extrusion head and melted inside a heated liquefier before being extruded through a fine nozzle, forming a single layer on the building platform. Each layer represents a cross-section of the 3D model. Upon extrusion and deposition of several layers on top of one another, the material solidifies and eventually forms the 3D object.

Figure 2.12: Schematic diagram of FDM process and FDM parameters, including slice thickness(ST), road width (RW), and raster gap (RG)

Researchers have demonstrated the feasibility of utilizing FDM to fabricate TE scaffolds directly. Zein *et al* used FDM to fabricate PCL scaffolds with honeycomb-like structure and channel sizes of 160 - 770 μ m at porosity ranging from 48 - 77% . According to Ramanath *et al* , the melt flow behavior of PCL can significantly affect the quality of the scaffold fabricated via FDM. This is dependent on physical properties like melt temperature and rheology, and also system properties like pressure gradient, velocity and temperature gradient within the nozzle. Another group of researchers has successfully extruded polypropylene/tricalcium phosphate (PP/TCP) scaffolds that consist of several concentric cylinders of different pore sizes . By varying the raster gaps and lay-down patterns for each concentric ring, it is possible for the resulting scaffold to consist of discrete rings each with a different porosity in the radial direction (see Figure 2.12). The drawbacks of the FDM technique include the need for the input material to be at specific diametric size and material properties to feed through the rollers and nozzle. The high operating temperature may also restrict the use of temperature sensitive materials.

Figure 2.13: Scaffold with porosity gradient in radial direction fabricated by FDM

2.3.2.2 3D Bioplotting

3D Bioplotting was developed at the Freiburg Materials Research Center in 2000 . The machine consists of a dispenser, which is movable in all three dimensions. The plotting material is stored in a cartridge and then plotted through a nozzle by compressed air. In this RP system, the key feature is the 3D dispensing of liquids and pastes in a liquid media having the same density of that of the scaffold material. The liquid medium compensates for the gravitational force and hence no support structure is needed. Upon leaving the nozzle, the plotting material solidifies in the medium after bonding to the previous layer. This RP process is reported to be capable of utilizing a wide range of scaffold materials which is an advantage in terms of material versatility . Unfortunately, the literature concerning the use of the bioplotting process for tissue engineering applications has been rather limited thus far .

2.3.2.3 3-Dimensional Printing (3DP)

3DP is a powder-based RP system that incorporates liquid binder for the purpose of fusing the powder particles (which can be polymer or ceramic powders) together in order to form a 3D object . The system consists of a building platform, which is movable in the z-direction, a roller to spread the powder onto the platform and a printhead which moves in x-y directions to dispense the liquid binder. In the building process, a stream of adhesive droplets is expelled, selectively bonding a thin layer of powder particles to form a solid layer. After one layer is complete, the platform will

lower down by a distance of a layer thickness and fresh powder will be spread onto the platform by the roller to cover the previous layer .

The commonly used polymer powders in 3DP process are PLA, PGA and PLGA . Kim *et al* employed 3DP with a particulate leaching technique for creating porous scaffolds using PLGA mix with salt particle and a suitable organic solvent. In an effort to render the system more biocompatible, Lam *et al* have formulated a blend of starch-based polymer powders that can be bonded using distilled water . Lee *et al* attempted an indirect 3DP approach, where the molds were first printed and the final scaffold material was cast into the mold cavity. Upon removal of the mold, the resulting PLGA scaffold consists of micro-villi features of 500 μm in diameter and 1 mm in height (see Figure 2.13).

Figure 2.14: Indirect 3DP process showing the mold and the final PLGA scaffold

2.3.2.4 Selective Laser Sintering (SLS)

SLS is a powder based RP system that uses a CO₂ laser beam to fabricate a 3D part. The building process is largely similar to 3DP in the sense that it uses a roller to spread fresh powder onto the part bed once a layer has solidified. The SLS machine uses a deflected CO₂ laser beam to selectively scan the cross sectional area of the part over the powder surface, using high temperature to temporally melt the powder particles and fusing them together.

As SLS involves fusing the powder particles using laser power, one of the challenges in using SLS to fabricate scaffolds is investigating how the laser affects the properties of the scaffold materials. The scaffold materials have to be carefully selected so that they do not degrade when sintered by the laser beam. By varying SLS parameters such as laser power, laser scan speed and part bed temperature, Tan *et al* has successfully sintered polyetheretherketone-hydroxyapatite (PEEK/HA) powder blends on a commercial SLS machine . Besides these system parameters, the particle size of the powder can also affect the accuracy and surface finish of the final scaffolds. It was suggested by Williams et al that the optimal power size should be in the range of 10-100 μm . More recently, PCL scaffolds and Poly(vinyl-alcohol)-hydroxyapatite (PVA/HA) composites have been fabricated for applications in bone regeneration using SLS . An advantage of SLS over FDM is the absence of support structures for overhanging features in the SLS process. Thus, post-processing of SLS fabricated scaffolds is much simpler as the powder that supports the overhanging features can be easily removed using compressed air to blow away the loose powder. Some limitations of SLS includes the low resolution of 300-400 μm and the high temperature involved in the process, which hinders the use of temperature sensitive materials.

2.3.2.5 ModelMaker II (MMII)

This system uses a single jet each for a plastic build material for the model and a wax-like support material for over-hanging features. The printer head ejects droplets of the materials as they are moved in a horizontal X-Y fashion. A milling head is passed over each layer to ensure that a uniform thickness has been achieved. The schematic diagram of the MMII process is shown in Figure 2.14.

MMII is not able to build scaffolds directly due to the limited availability of raw materials, which are limited to only Solidscape's ProtoBuild™ and ProtoSupport™. By employing the indirect scaffold fabrication approach, a wider range of materials

Figure 2.15: A schematic diagram of MMII process

can be used to produce scaffolds using MMII. Taboas *et al* successfully produced PLLA scaffold with local and global porosity for bone tissue engineering, specifically the trabecular bone. The mold (see Figure 2.15) was built using ProtoSupport™, which is a wax material and can be removed by melting the wax or by dissolution in suitable solvents. Using a similar approach, Limpanuphap and Derby fabricated tricalcium phosphate (TCP) scaffolds with controlled internal porosity with a suspension of TCP in acrylate binder. In recent developments, collagen scaffolds with internal channels have been fabricated by Sachlos *et al* and Yeong *et al* using the MMII. As compared to synthetic scaffolds that have been fabricated using the RP technologies discussed so far, collagen offers a much more native surface for cell-scaffold interaction since it is the major component of the ECM.

Figure 2.16: ProtoSupport™ Mold with struts branching in x, y and z directions

2.3.2.6 Stereolithography (SLA)

In this liquid based RP system, a UV laser traces out the layer on the surface of a vat of photocurable resin, solidifying the model's cross-section while the areas which are not a part of the model remain liquid (see Figure 2.16). The elevator then drops enough to cover the solid polymer with another layer of liquid resin and the laser traces the second layer atop the first.

Figure 2.17: Schematic diagram of SLA process

SLA can be used to fabricate scaffolds directly. Arcaute *et al* encapsulated human dermal fibroblasts in bioactive PEG hydrogels that were photocrosslinked using STL. Cell viability was reported to be 87% at 24 hours after fabrication. Using the indirect approach, Chu and Halloran have successfully produced HA-based porous implants using SLA-built epoxy molds. The mold was removed by pyrolysis after which the remaining cast was sintered to obtain HA scaffolds with sufficient mechanical strength. The resolution of channel width achieved was as low as 366 μm . The same group of investigators carried out an *in vivo* study using two different architectural designs, orthogonal and radial channels. Preliminary results showed that controlling the overall geometry of the regenerated bone tissue was possible through the internal architectural design of the scaffolds. In another study, Kang *et al* fabricated water soluble molds which have interconnected struts of around 100 μm using STL. Upon casting of the scaffold material, the resulting PCL scaffold consists of interconnected channels which were tested to be non-cytotoxic to cells.

2.3.3 Electrospinning

Electrospinning is a well-established method for fabricating nanofibrous scaffolds with high porosity. Due to their resemblance scale wise to native ECM, this scaffold fabrication technique has been extensively researched in recent years. The process involves a high voltage field that is created between a syringe containing the polymer solution and a collector. As streams of polymer fluid are ejected from the syringe, it undergoes a whipping process due to the electric field and forms a charged polymer fiber that lands on the collector. The diameter of the fibers can range from hundreds of nanometers to several micrometers, closely mimicking the native extracellular protein fiber diameter. By using a rotating mandrel as the collector, the collected fibers can take the shape of the mandrel, forming a tubular polymeric fibrous mesh. Scaffolds fabricated using electrospinning technology are characterized by their small mesh sizes with densely packed morphology. It is also possible to guide cellular orientation through contact guidance by aligning the direction of the fibers.

Researchers have studied different aspects of the electrospinning process to determine how process parameters affect the properties of the electrospun scaffolds. Generally, the alignment of fibers can be controlled in a preferred direction by adjusting the rotation speed of the collector. This was effectively demonstrated by Thomas *et al* who also observed an improvement in tensile strength and modulus of electrospun PCL meshes. Similarly, Li *et al* reported greater tensile strength at high rotation speeds due to increased fiber alignment and packing. The concentration and the flow rate of the polymer solution are also known to affect uniformity and diameter of the fibers. Vaz *et al* demonstrated the versatility of the electrospinning process by using a sequential multilayering technique to create an outer aligned fibrous PLA layer and a randomly oriented inner PCL layer for engineering blood vessels.

An important issue in using electrospun scaffolds for tissue engineering is the degree of cell infiltration across the scaffold. To engineer large tissues like the liver and heart muscles, the infiltration of cells into a 3D framework is critical. However, electrospun scaffolds are generally known to be associated with limited cell infiltration due to

their characteristically high fibre density and small pore sizes (less than 10 μm) . With little to no infiltration, the bulk of the scaffold interior becomes devoid of cells and ultimately devoid of any potential to serve as a functional tissue replacement. Some strategies have been adopted by researchers to alleviate this problem of limited cell infiltration. For example, Leong *et al* modified the electrospinning process by incorporating an additional cryogenic setup. This setup allows the creation of an electrospun scaffold with an open-pore morphology through the formation of ice crystals and its subsequent sublimation. Such scaffolds with open-pore structure can better facilitate the ingrowth and migration of seeded cells and also enhance mass transfer. A different approach involves the simultaneous electrospinning of fibers and living cells to achieve a homogenous cell distribution across the entire scaffold thickness . Studies have shown that the process did not affect the viability of the cells and they remained viable over long term. Although this approach may effectively solve the problem of cell infiltration, it requires careful selection of cells, scaffold materials and integration with the spinning process in order to ensure cell viability and to avoid cellular damage. Thus, the feasibility of using electrospun scaffolds for large tissue regeneration is still being studied. Nonetheless, electrospinning continues to be an attractive scaffold fabrication technique due to their process versatility with both synthetic and biological materials.

2.4 Challenges in Scaffold Fabrication

One of the challenging aspects of tissue engineering, in addition to scaffold design, is to develop suitable fabrication method(s) to produce scaffolds that will meet the essential design requirements. The physical design, defined by its macro- and micro-architecture (e.g., porosity, pore size, permeability, pore interconnectivity, scaffold geometry) is known to extensively influence the transport efficiency of nutrients as well as the behavior of cells within the scaffold. These features are in turn, largely determined by its fabrication process. Thus, the fabrication method must address controllability of scaffold architecture at both the micro- and macro-scale levels. The fabrication process must also be compatible with the material intended for use as the scaffold.

2.4.1 Limitations of Conventional Fabrication Methods

In the tissue engineering process, the selected scaffold fabrication method must be able to control both micro-scale features such as pore size, pore interconnectivity, and macro-scale features such as porosity, geometry and the overall internal configuration e.g., presence of interconnected channels. In general, most of the fabrication methods discussed thus far fulfill only one of these two requirements of scaffold fabrication. Conventional methods have been widely reviewed and are generally known to produce bulk, foam structures with random pore architectures e.g., non-uniform pore sizes and/or random pore interconnectivity. However, these techniques are still commonly used today for scaffold production because of their relative simplicity and also without the need for any expensive and specialized equipment. Nonetheless, it is often difficult to control the fabrication process e.g., the growth and nucleation of CO₂ gas or the rate of ice crystallization in phase separation, gas foaming techniques etc. Thus, the resulting porous structures of these scaffolds are highly process driven and in most cases, with non-homogenous and poorly controlled pore architectures. As a result, the degree of control over scaffold pore structure offered by these fabrication methods is very limited. In the solvent casting/particulate leaching process, it is difficult to control the distribution of the porogens in the scaffold material solution. The natural dispersion of the porogens results in very minimal contacts between one another and this creates un-interconnected or closed pores upon porogen leaching. Two possible explanations occur as to why contacts between porogens are too limited to provide any significant interconnection:

- a) The shape of the porogens is irregular in nature. This prevents them from achieving a closed packed structure due to their random nature of stacking, thus forming dead end spaces with only a single contact to neighboring porogens. Consequently, this restricts end-to-end interconnectivity between pores (see Figure 2.17). In a study by Draghi *et al*, the fabrication process of PLA scaffolds using a variety of porogens including salt crystals, gelatin microspheres and paraffin wax microspheres was investigated. The study showed that highly permeable scaffolds were created when microspheres were used as the porogens, thus highlighting

the improved pore interconnection as compared to those obtained using irregular-shaped salt crystals.

Figure 2.18: A small section of the scaffold illustrating pores with only a single opening

- b) There is insufficient necking/fusion between individual porogens. Upon casting the polymer, they disperse randomly in the solution and contacts are minimized. Even if contacts exist between the porogens, they are ‘just touching’ each other and the contact between one another is very minimal. Thus, some form of fusion must occur between the porogens in order to increase the area of contact, and hence the interconnection between pores.

The reverse templating process seems most promising as it creates necking between the porogens during the sintering process. Like the other fabrication methods however, the scaffolds fabricated by this technique are limited to simple geometries, which may not fit well into the defect shape of the tissue/organ. Furthermore, it is unclear as to whether scaffolds of large sections can be fabricated due to the difficulty

of casting the scaffold material into thick template sections. In short, conventional methods of fabrication rely heavily on user skills and experience; therefore, the microstructure of the resulting scaffolds is usually not consistent and not readily reproducible .

2.4.2 Resolution and Material Limits of RP Techniques

RP fabricated scaffolds are generally characterized by their 3D, open and highly interconnected structures. In contrast to conventional scaffold fabrication methods, RP techniques offer users sufficient control over the internal design of the scaffold. The ability to construct scaffolds with intricate architectures (see Figure 2.18) in a reproducible manner is one of the key advantages offered by these processes . Such versatility allows scaffolds with complex architectures and geometry e.g., multi-layered scaffolds, interconnected channel features or tubular shaped scaffolds to be designed and constructed in a CAD/CAD fashion. However, even with greater control over the internal design of the scaffold, limitations still exist in using RP techniques for scaffold fabrication.

Figure 2.19: FDM fabricated PP-TCP composite scaffolds

In general, RP fabricated scaffolds tend to have lower porosity compared to those fabricated via conventional methods. This can be explained in terms of the resolution limits of the RP systems. Taking FDM for example, the achievable resolution as reported by Yeong *et al*, is relatively low at 250 μm . The filament extruded during the FDM process is usually non porous and the raster gap between the filaments is small. As a result, the overall void spaces within the final scaffold construct is very limited, indicating a low porosity. Using FDM, Kalita *et al* fabricated PP-TCP

scaffolds with porosity values ranging from 36 to 52% (see Figure 2.18). For SLS, the smallest feature that can be fabricated is as large as 360 μm due to the laser spot size. In 3DP process, the achievable resolution reported to be 300 μm . Kim *et al* employed 3DP to fabricate PLGA scaffolds having pore sizes in the range 45–150 μm with 60% porosity. Aside from the characteristically low porosity of RP scaffolds, the resolution limits of these RP systems would also mean that they would be unable to reliably produce micron-sized features e.g., pore size. Similar to direct RP processes, the indirect RP approach has a high degree of control over the internal architecture of the scaffold, being able to fabricate scaffolds with complex channel configurations. However, indirect RP processes are still unable to control the pores of the scaffolds, often resulting in random pore sizes and shapes. Kang *et al* used an alkali-soluble mold fabricated via SLA to produce PCL scaffolds with interconnected channels (see Figure 2.19). Although the channel networks were visibly well-integrated within the scaffold, the scaffold pores

Figure 2.20: PCL scaffolds with interconnected channels fabricated using indirect SLA process

appeared to be of random sizes and randomly distributed. Similarly, Sachlos *et al* fabricated collagen scaffolds with internal channels using the indirect approach. However, the pore structure was inconsistent and there appeared to be skin layers at the interface between the channels and the local regions (see Figure 2.20).

Figure 2.21: Collagen scaffolds with intricate channel features fabricated from MMII mold

In addition, RP was originally conceived to produce engineering prototypes rather than bio-functional structures. Due to the nature of their fabrication approach e.g., the elevated temperatures and toxic solvents involved in a number of RP processes, most biomaterials may not be compatible with these systems. The potential scaffold materials that can be used for scaffold fabrication are therefore limited. To date, the biomaterials that have been used in conjunction with currently available RP systems are limited to synthetic polymers such as PLGA, PLA, Polyvinylalcohol (PVA), Polycaprolactone (PCL) and ceramics such as Hydroxyapatite (HA) and tri-calcium phosphate (TCP) etc. . Such limitation has prompted research in the direction of adopting alternative approaches to RP fabrication. For example, Yeong *et al* managed to produce collagen scaffolds with predefined internal architecture through the indirect fabrication approach involving the use of a sacrificial mold printed from MMII system. Pham *et al* applied the concept of rapid freeze prototyping in the bioplotting process and successfully fabricated chitosan scaffolds for tissue engineering applications. Such techniques could potentially broaden the scope of biomaterials that can be used for RP fabrication, though this require time consuming, back and forth testing and additional machinery to identify systems parameters that are compatible with these scaffold materials. Likewise, increasing the resolution of

these RP systems may also require customization of the RP system such as changing system settings as well as installing additional parts which may be costly, knowledge/skill dependent and also time consuming e.g., new lens for changing the laser spot size for SLS and smaller diameter nozzles for the FDM process.

2.5 Summary

The selection of a compatible scaffold material and employing a suitable fabrication method remains challenging. In terms of structural control, no single current fabrication method seems to be able to sufficiently control every aspect of the scaffold pore structure. Thus, the need to develop novel fabrication techniques that encompass a wider range of materials and simultaneously allow sufficient control over the micro- and macro-scale features of the scaffold is necessary. It is believed that this can be achieved through the integration of different scaffold fabrication techniques into a single process. In this manner, the advantages of individual techniques can circumvent the disadvantages of one another and vice versa.

Current RP fabrication methods have greatly aided in overcoming some of the limitations of conventional scaffold fabrication methods. Among the three major fabrication categories discussed, RP methods was shown to be the more promising scaffold fabrication approach in terms of controllability. However, while the versatility of RP techniques in controlling macro-features (e.g, geometry, interconnected channel features) is well recognized, it is highly unlikely that RP alone will be able to meet all the design requirements of a scaffold. This is because the current resolution of most RP systems operates on a scale of 100 μm or larger . Considering that the length scales of structures that govern several cell-specific functions such as adhesion, proliferation and differentiation are in the order of tens of microns, current RP technologies may not be feasible in producing scaffolds that can sufficiently control these functions . If micro-scale characteristics such as pore size, distribution and pore interconnectivity are to be precisely controlled as well, other fabrication methods that offer sufficient micro-architectural control (e.g., microsphere leaching) may have to be used in conjunction with RP technology. As of now, RP is

more appropriate for constructing macro-scale features such as channel networks which are required in large scaffold sections.

The electrospinning process and conventional fabrication methods possess a common disadvantage: they are limited to simple geometries. Although the pore structure of scaffolds fabricated from electrospinning is touted to be almost similar to the native ECM structure, the scaffolds are sheet-like and too thin to be used for 3D tissue regeneration. The particulate/leaching and reverse templating method allows the tissue engineer to control the size and shape of the pores through careful selection of the type of porogens used. To achieve a highly interconnected pore network, porogens with fixed, regular shape must be employed in the process so as to maximize the contact between individual porogens. From this viewpoint, microsphere particles can be a viable alternative to other forms of porogens such as salt and sugar crystals since they are completely spherical in shape. The incorporation of a sintering process to fuse the microspheres together can also effectively increase the area of contact between one another, and thus, pore interconnectivity. The ability to precisely control the size of pores may be crucial in studying certain cell behavior and can also function as an investigative platform for research on cell-cell, cell-scaffold interactions.

Preferably, RP techniques are best used for macro-structural control whereas the reverse templating method can be used to control the size of micro-pores. To incorporate a wider range of scaffold materials and to avoid time consuming, costly customization/development of RP systems, the indirect RP approach should be adopted. The indirect RP process, combined with reverse templating technique can be a promising, alternative way to prepare scaffolds with tailored pore structures and complex channel features.

Chapter 3 – INVERSE SCAFFOLD FABRICATION PART

I: RAPID PROTOTYPING PROCESS

The proposed inverse scaffold fabrication method requires the use of a mold in which its design corresponds to the negative replica of the entire scaffold architecture. This mold comprises of two components: the first being a RP fabricated structure that will be filled with micro-particles, which are the second component. Upon the dissolution of the mold, the RP structure will shape the macro-features e.g., interconnected channels, geometry etc., while the micro-particles form the micro-pores of the resulting scaffold. This chapter focuses on the RP aspect of the mold fabrication process. An insight study on the PolyJet™ printing process, specifically the Objet 3D printer (Eden 350V) is presented, so as to better understand the fundamentals of the process. The study forms the basis for the investigation of specific variables and their effects on the process and prediction in printing performance. Moreover, since the printer is originally designed for fabrication of large models, it is necessary to determine its suitability for the fabrication of small channel structures as required in this research. The performance of the printer is important in defining the ability to achieve the predetermined dimensions of the RP mold to be used in the inverse scaffold fabrication method.

3.1 Background on Inkjet Technology and Three Dimensional Printing

Inkjet printing is an important technology owing to its adaptability in various applications. These include spray coating , microarray drug testing and DNA patterning , direct writing and packaging , manufacturing and more recently, rapid prototyping . The most common approach utilized in inkjet printing technology is the drop-on-demand inkjet. As its name implies, drop-on-demand printers eject a drop of ink from the nozzles only when required. To trigger the ejection, two common technologies are available: bubble jet and piezoelectric ejection.

3.1.1 Bubble Jet Printing

In bubble jet printing, a pulse current heats a resistor to a high temperature within microseconds, creating a vapor bubble on the surface of the heater (see Figure 3.1). This bubble causes a droplet of ink to be ejected from the nozzle. The vapor bubble then cools and collapses, allowing the ink chamber to be refilled from the reservoir. The main problem with bubble jet printers is the high frequency of nozzle failure as a result of the high temperature required for rapid drop ejection. This causes decomposition of ink component on the resistor, which leads to poor heat transfer and nozzle clogging. Overall, the bubble jet offers low cost print heads but is compromised with low reliability.

Figure 3.22: Bubble jet printing

3.1.2 Piezoelectric Ejection

Today, piezoelectric ejection is the most common method of ejection used in inkjet printers. The operational principle of piezoelectric drop-on-demand inkjets is the ejection of droplets by the actuation of a piezoelectric material using voltage pulses. The resulting expansion and contraction of the piezoelectric in the ink chamber creates a pressure wave that travels through the ink into the nozzle bore. This causes the ejection of a specific volume of fluid from the nozzle (see Figure 3.2). Droplet positioning is achieved by moving the printhead to the desired location above the substrate before drop ejection. On completion of the pulse cycle, the piezoelectric element returns to its normal dimensions and the ink chamber is filled from an ink

reservoir by capillary action. The absence of heat in this mode of ejection allows a longer life span of the printhead and higher reliability than the bubble jet method.

Figure 3.23: Piezoelectric printing

3.1.3 Piezoelectric Technology in Rapid Prototyping

Aside from being used in inkjet printers, the piezoelectric printing technology was also adopted in rapid prototyping systems to fabricate 3D models (e.g., Model maker II, Objet printers), which is commonly addressed as three dimensional printing (3DP). The droplets deposited from these RP systems are such that it can be solidified and formed into a 3D part on a layer by layer basis. Similar to inkjet printing, these RP systems function on an individual droplet level. It operates by firing droplets from a piezoelectric printhead onto an adjustable platform. Each individual droplet impacts the substrate and spreads into a spherical cap defining the resolution of the machine. By controlling the spacing between droplets, a straight line is formed as the droplets coalesce while the printhead transverses across the platform. The width of the line depends on the spacing between droplets as well as the spreading kinetics of each individual drop. Several lines form the layer creating the cross section of the model being built. After a single layer has solidified, the ejection of droplets onto the previous layer continues in a layer by layer fashion until the model is completed. A

schematic representation of a droplet based fabrication process is presented in Figure 3.3.

Figure 3.24: Schematic representation of droplet-based fabrication process

3.2 Objet's PolyJet™ Printing Process

As a hybrid between 3DP and SLA techniques, the PolyJet™ printing technology is a raster scanning, layer-additive fabrication technique that is employed in all Objet printer models. It utilizes piezoelectric technology to deposit photocurable polymers as raw materials and is capable of producing results similar and much faster to the considerably more expensive SLA machines. Combined with multi-nozzles printheads, prototypes can be fabricated in a relatively accelerated pace using Objet. Overall, the PolyJet™ printing technology is a unique, delicate printing process involving the precise deposition and photocuring of micron-size photopolymer droplets .

3.2.1 Printheads

Similar to most RP systems, Objet fabricates complex 3D designs from CAD data on a layer by layer basis. The CAD model of the 3D object to be built is processed in a computer where it is sliced into many individual layers, each depicting a single cross section of the model (see Figure 3.4).

Figure 3.25: Part divided into several layers each depicting the cross section of the part

Instead of single printhead used in FDM and MMII, eight jetting heads with 96 nozzles in each head simultaneously deposit identical amounts of photopolymer droplets onto the build tray. The photopolymers are stored in cartridges and are fed to the individual printheads via feedlines by pumps. Upon actuation of the piezoelectric element, a series of acoustic waves form in the fluid chamber which propagate towards the nozzles. Ejection of a droplet occurs when the energy level at the nozzles exceeds the surface energy of the liquid as a result of the pressure waves. The droplet travels a short distance and impacts on the substrate, causing it to spread into a shape of a spherical cap. At the appropriate actuation frequency and printhead traveling speed, droplets will be deposited in a successive manner and coalesce to form a straight line prior to solidification. The mode of solidification employed in PolyJet™ printing utilizes ultra violet (UV) bulbs which are located alongside the printheads to immediately cure each layer after a single pass of the printhead (see Figure 3.5).

Figure 3.26: UV lamps attached to the printhead curing the layer as it traverses along the build tray

Among the 8 printheads, 4 are used for jetting model materials and the other 4 for support materials. It employs a raster scanning process by mechanically traversing the printhead along the build tray at high speed to print both the model and support material simultaneously in order to form a single layer. This enables the printer to build in 65mm wide slices instead of a point by point approach. It is important to note that since the printhead only traverses in the x-axis, the lines deposited on the platform are always parallel to one another and along the printhead scanning direction. Thus, each layer is composed of several parallel lines forming a single slice which illustrates a cross sectional image of the 3D part (see Figure 3.6).

Figure 3.27: Several parallel lines form the image of the cross section of the part

3.2.2 Printing Sequence

The printing system adopted by the Objet 3D printer employs an alternate line printing scheme. In the first pass of printing, droplets are jetted onto the substrate, forming alternate rows of lines as the drops spread and coalesce (see Figure 3.7). On the return pass, the printhead is offset in the y-axis. As it transverses back to the starting position, printing resumes by jetting out droplets to fill the space between the printed lines that are created in the forward pass, creating a continuous thin layer on the substrate (see Figure 3.8). If the cross section of the image is wider than 65mm, a second or more passes will be required to complete the entire layer. Once a layer has been completed, the substrate is lowered in the Z-axis by a layer thickness and the printing continues to jet the next layer on top of the previous one. Such a printing scheme relies on the accurate placement of droplets on the substrate and the precise servo control between the printhead and the motor. The distance between the impact positions of droplets must be controlled as the film thickness is determined by the numbers of droplets per unit area. The deposited droplets in the return pass must also be able to fill the gaps between the alternate lines formed in the forward pass in order to form a continuous layer. As such, the distance between nozzles and the spreading kinetics of droplets are critical parameters affecting part accuracy as they define the width of individual printed lines and the separation distance between lines.

Figure 3.28: Alternate lines printed on the substrate during the forward pass

Figure 3.29: Alternate line printing scheme

3.2.3 Overview of 3D Model Fabrication

The entire fabrication process of a 3D model is briefly illustrated in Figure 3.9. First, the desired model to be fabricated is designed using a commercial CAD software e.g., SolidWorks, ProEngineer etc. Prior to fabrication, the CAD data, which is exported as a .STL (stereolithography) format file from the CAD software is then processed by Objet Studio™, the proprietary software of Objet geometries Ltd. This software slices the CAD data into several cross sections which are then individually sent to the printer computer. Together, these cross sections form the entire 3D model. Objet Studio™ also offers simple commands such as enlargement, flattening or reduction of the model size and also allows users to choose the orientation in which the model is to be fabricated. Once the CAD data preparation is completed, the 3D model is ready to be fabricated from the Objet printer.

Figure 3.30: Overview of fabrication process of a 3D model

For this research, Fullcure®720 and Fullcure®705, the proprietary model and support materials respectively, are used for the fabrication. Upon completion of the printing process, the 3D model is detached from the build tray and subsequently processed with 1M sodium hydroxide (NaOH) in order to remove the support material. This process is carried out by soaking the 3D model in a beaker of NaOH overnight. The model is then repeatedly rinsed with water to remove traces of NaOH.

Two printing modes are available for the Objet printer: quality mode and speed mode. Although both modes use the alternate line printing scheme, there are differences in the printing process of these two modes. These will be discussed in the following sections.

3.2.4 Kinetics of Droplet Spreading

The impact of a liquid droplet on a solid surface is a phenomenon involved in numerous engineering applications e.g., film coating, solder deposition, spray cooling, and inkjet printing. A droplet in contact with a substrate may spread and adopt the

shape of a truncated sphere. Depending on the droplet momentum and substrate properties, it may also experience splashing in which the drop breaks up into several satellite droplets or in severe cases, they may even bounce off the substrate surface . In both printing modes, photopolymer droplets of diameter d are deposited on the build tray and spread into a shape of a spherical cap with a base diameter of D_{max} . The spreading of a single droplet affects the formation of a single line and thus the overall resolution of the Objet printer. A theoretical approach in predicting the spread phenomenon is based on the conservation of energy and involves the use of an energy conservation equation on the system. Referring to Figure 3.10, the system energy at the instance of impact (state 1) is set to be equal to the energy at maximum spreading state (state 2) (see Figure 3.11). From the balance of energy:

$$E_{k1} + E_{SL1} = E_{k2} + E_{SL2} + E_{SL2} + E_{V2} \quad (\text{Equation 3.1})$$

where subscripts 1 and 2 represent the states at the instance of impact and at maximum spread respectively. On the left side of the equation, E_{k1} refers to the kinetic energy at the instance of impact and E_{SL1} is the surface energy of the droplet.

Figure 3.31: From droplet impact (state1) to maximum droplet spread (state2)

On the right hand side, E_{k2} and E_{SL2} are the kinetic energy and surface energy of the droplet in contact with the surrounding vapor at maximum spread respectively. E_{SL2} refers to the interfacial energy between the drop and the solid substrate. Finally, E_{V2} is the energy loss through viscous dissipation in the droplet as it deforms from a sphere into the shape of a spherical cap. These terms can be expressed as follows:

$$E_{k1} = \frac{\pi}{12} \rho d^3 U^2 \quad (\text{Equation 3.2})$$

$$E_{SL1} = \pi d^2 \gamma_{LV} \quad (\text{Equation 3.3})$$

$$E_{SL2} = \pi \gamma_{LV} (D_{\max} h + D_{\max}^2 / 4) \quad (\text{Equation 3.4})$$

$$E_{SL2} = \frac{\pi}{4} D_{\max}^2 (\gamma_{SL} - \gamma_{SV}) \quad (\text{Equation 3.5})$$

where h is the height of the droplet at maximum spread, assuming that the spread droplet adopts the shape of a cylindrical disk. ρ is the density of the droplet, U is the

Figure 3.32: Individual photopolymer droplets (state 2) collected from the Objet printer A) Top view (80X), B) Side view (500X)

impact velocity of the droplet and γ_{LV} , γ_{SL} , γ_{SV} are the surface tension at the liquid/vapor, substrate/liquid and substrate/vapor interface respectively. The kinetic energy E_{k2} at maximum spreading position is zero. Finally, the work done against viscous dissipation E_{V2} as the droplet deforms from state 1 to 2 will be discussed next.

Energy loss due to viscous dissipation

The work done in deforming the droplet against viscosity is given by Chandra *et al* :

$$E_{V2} = \int_0^{t_c} \int_V \phi dV_{drop} dt \approx \phi V_{drop} t_c \quad (\text{Equation 3.6})$$

where ϕ is the viscous dissipation function, V_{drop} is the volume of the fluid and t_c is the time taken for the droplet to reach maximum spread. Dissipation function ϕ for a Newtonian fluid can be approximated by:

$$\phi = \mu \left(\frac{dU}{dY} \right)^2 \approx \mu \left(\frac{U}{L} \right)^2 \quad (\text{Equation 3.7})$$

in which L is the characteristic length of the spreading droplet in the y direction and μ is the viscosity of the droplet. Chandra *et al* who assumed the spreading droplet to be that of a cylindrical shape used the thickness of the cylindrical droplet as the characteristic length L . This results in over- prediction of maximum spreading ratio β

, which is $\frac{D_{max}}{d}$ and suggests that the characteristic length L is actually smaller than

the cylindrical thickness. A more appropriate characteristic length proposed by Pasandideh-Fard *et al* is to use the boundary layer thickness, δ , to estimate the magnitude of viscous dissipation. According to Pasandideh-Fard *et al*, the motion of the droplet as it spreads can be represented by an axisymmetric stagnation point flow. The boundary layer thickness is given as :

$$\delta = 2 \frac{d}{\sqrt{Re}} \quad (\text{Equation 3.8})$$

The height of the droplet at maximum spread will be that of the boundary layer. Hence, the volume of the droplet at maximum spread can be calculated using this expression:

$$V_{drop} = \frac{\pi D_{max}^2 \delta}{4} \quad (\text{Equation 3.9})$$

The time taken to reach maximum spread diameter t_c can be represented by using the simple assumption that the drop spreads into a cylindrical disk of increasing diameter D and thickness h (see Figure 3.12). By the conservation of mass, the velocity at the edge of the spreading drop is:

$$\frac{V_R}{U} = \frac{a}{4Dh} \quad (\text{Equation 3.10})$$

Equating the volume of a sphere to that of a cylindrical disk and rearranging gives:

$$h = \frac{2d^3}{3D_{max}^2} \quad (\text{Equation 3.11})$$

The value of a varies between 0 and d during impact. Taking an average value of $d/2$ and combining these two equations results in:

$$V_R = \frac{3}{16} U \frac{D_{max}^2}{d} \frac{1}{D} \quad (\text{Equation 3.12})$$

where $V_R = \frac{dR}{dt} = \frac{1}{2} \frac{dD}{dt}$. Finally, integrating 3.12 gives the expression:

$$\frac{D}{D_{max}} = \sqrt{\frac{3}{8} t \frac{U}{d}} \quad (\text{Equation 3.13})$$

From this equation, the time taken to reach maximum spread $D = D_{max}$ will be

$t_c = \frac{8}{3} \frac{d}{U}$. Thus, substituting $t_c = \frac{8}{3} \frac{d}{U}$, Equation 3.7 to 3.9 into Equation 3.6 gives:

$$E_{V2} = \frac{1}{3} \pi \frac{\rho U^2 d}{\sqrt{Re}} D_{max}^2 \quad (\text{Equation 3.14})$$

Figure 3.33: Spreading into a cylindrical disk of thickness, h

Deriving the maximum spread ratio

The relationship between the three forces of interfacial tension of each participating phase (vapor, liquid, solid) can be described by Young's Equation:

$$\gamma_{LV} \cos \theta = \gamma_{SV} - \gamma_{SL} \quad (\text{Equation 3.15})$$

Substituting the relations (Equations 3.2, 3.3, 3.4, 3.5, 3.14) into Equation 3.1 and

applying Young's Equation and the definition of Reynold's number, $Re = \frac{\rho U d}{\mu}$ and

Weber's number $We = \frac{\rho U^2 d}{\gamma_{LV}}$, we obtain:

$$\beta = \sqrt{\frac{We + 12 - \frac{8}{\beta}}{3(1 - \cos \theta) + 4 \frac{We}{\sqrt{Re}}}} \quad (\text{Equation 3.16})$$

Where θ is the contact angle of the droplet with respect to the solid substrate at equilibrium, which is measured to be 19.9° using the drop shape method. The diameter of the droplet at maximum spread can be calculated from the spread ratio β

$= \frac{D_{\max}}{d}$. Thus, an analytical model based on energy balance approach incorporating the effects of droplet size, impact velocity, surface tension and viscosity is developed to predict the maximum spread diameter of a single droplet.

3.2.5 Line Formation

To form a continuous line, the positions of the photopolymer droplets have to overlap each other in a successive manner in order to coalesce with one another. The distance between adjacent droplets is controlled by the scanning speed of printhead S , as well as the frequency of droplet ejection, f . As the printhead traverses across the building tray, droplets will land with a center-to-center separation C (see Figure 3.13). This can be represented by the correlation:

$$C = \frac{S}{f} \quad (\text{Equation 3.17})$$

where C is the distance from the center of the droplet to that of the adjacent droplet.

Using Equation 3.16, the width of a printed line can be derived from the spread diameter of a single droplet. When droplets land on the build tray with a center-to-center separation distance C , the physical boundary of each individual spread droplet overlaps the adjacent droplet and may also overlap the neighbouring droplets if the spread diameter is large enough (see Figure 3.13). Thus, the actual volume at any point of the line can be a summation of several droplets depending on the spread diameter and the distance C .

To calculate the line width, the total volume contributed from the neighboring droplets that are enclosed within the physical boundary of an individual spread droplet is assumed as one single drop of volume V_L with a new drop diameter, d_L . The dimension of this physical boundary is assumed as the spread diameter of one single photopolymer droplet, which can be obtained using Equation 3.16. With a new drop diameter d_L , a new spread ratio β and hence, a new spread diameter D_L can be derived. This spread diameter is assumed as the width of a printed line.

Figure 3.34: Illustration of line formation involving overlapping droplets

In the speed mode, the value of C is much smaller due to the higher droplet frequency as compared to the quality mode. The total volume enclosed within the physical boundary of a spread droplet can be derived using simple geometrical analysis. According to the manufacturers specification (see Table 3.1), the center to center distance C is $42\text{ }\mu\text{m}$ in the quality mode and the layout of the droplets is illustrated in Figure 3.14. For simplicity, every spread droplets are assumed as a cylindrical disk. In the quality mode, the n th droplet is overlapped by the $(n-2)$ th, $(n-1)$ th, $(n+1)$ th and $(n+2)$ th droplets. For example, the illustration in Figure 3.14 assumes the third cylindrical disc as the point of interest to obtain the total volume. Therefore, the physical boundary of the third disc is overlapped by the first, second, fourth and fifth disc. Based on the manufacturers specifications (see Table 3.1), a single droplet

volume of 10 Pico-liters will result in a spread diameter of 110.9 μm . To calculate the total volume, V_L within the physical boundary which is 101.3 μm wide, the portion of each overlapping disc that is within this boundary is expressed as a percentage of a

Table 3.1: Manufacturer's printer and material specifications

	Quality mode	Speed mode
Droplet volume	10 Picoliters	
Polymer density (kg/m^3)	1189	
Surface tension (dynes/cm)	40	
Viscosity (Pa.s)	0.001	
Drop frequency, f (KHz)	10	20
Printhead speed, S (m/s)	0.42	0.38
Center to center distance, C (μm)	42	19

Figure 3.35: Illustration on the overlapping of cylindrical discs in quality print mode full cylindrical disc and multiplied by the volume of a single droplet before being added together. Based on this volume, and using Equation 3.16, the width of a printed line, W is 142.4 μm for the quality mode. For the speed mode, the center to center

distance of 19 μm results in a total of 10 overlapping droplets within the physical boundary and a resulting line width of 177.9 μm . It is important to note that the droplet velocity, U used in Equation 3.16 is an estimated value as it is not possible to obtain this value from the manufacturer and also due to the unavailability of suitable equipment that can be used to measure this value. However, this value is well within the range of inkjet printers that are documented in literature.

3.2.6 Layer Thickness

As discussed, the printing sequence of the Objet printer is an alternate line printing scheme. When the printhead has completed its forward pass, it is mechanically offset in the y axis before it undergoes its return pass. During the return pass, the printhead deposits photopolymer droplets to fill up the spaces between the alternate lines printed during the forward pass, thus creating a layer. The thickness of this layer determines the z resolution of the Objet printer.

Using mass conservation, the volume of the coalesced line is equal to the total volume of the droplets jetted out from the nozzles:

$$V_{drop} \times N_{drop} = A \times L \quad (\text{Equation 3.18})$$

where N_{drop} is the total number of droplets used to form the line, A is the cross sectional area of the line and L is the length of the line (see Figure 3.15). Knowing for

a given time t , $L = S t$, $N_{drop} = ft$ and $V_{drop} = \frac{\pi d^3}{6}$, the equation becomes:

$$\begin{aligned} \frac{\pi d^3}{6} ft &= ASt \\ A &= \frac{\pi d^3 f}{6S} \end{aligned} \quad (\text{Equation 3.19})$$

Figure 3.36: Cross sectional area of a printed line

The distance between each individual printhead nozzles will determine the separation distance between alternate lines printed during the forward pass. Together with the cross sectional area A , this line to line separation distance, D_L will establish the layer thickness, which is the z resolution of the Objet printer. If D_L is larger than two times the width of a line, W , the lines printed during the return pass are unable to coalesce with the ones printed during the forward pass (see Figure 3.16). As subsequent layers are deposited on top of one another, the part becomes inaccurate due to the unevenness in the layer thickness. Thus, the printhead design has a nozzle to nozzle distance, and hence D_L , that is less than $2W$. In this case, the lines formed during the returning pass are able to fill the gaps between the lines printed during the forward pass, thus resulting in a layer of even thickness (see Figure 3.17 for illustration).

The average line thickness printed via quality mode, as measured using a contact profilometer (Telescan 150) is $16.21 \mu\text{m}$ (see Figure 3.18). After the return pass, the layer thickness is measured to be $21.1 \mu\text{m}$. From the profile in Figure 3.18A, the peak to peak distance (the separation distance between lines D_L), is estimated to be $160 \mu\text{m}$. This was further verified by using a laser displacement meter to measure the offset distance that the printhead undergoes in the y axis just before the return pass.

Figure 3.37: Formation of uneven layer thickness ($D_L > 2W$)

Figure 3.38: Illustration of the formation of a single layer (cross sectional view) Blue: lines printed during forward pass. Red: lines printed during the return pass

The average offset distance is measured to be $80.3 \mu\text{m}$, which is half the separation distance D_L . From the profile, the line width, W is approximately $150 \mu\text{m}$, which is close to the theoretical value derived from Equation 3.16.

Figure 3.39: Profile of A) alternate lines, B) one layer obtained using a contact profilometer

In the speed mode, the profile measured using the profilometer shows a slightly higher line thickness and layer thickness of 27.3 μm and 36.14 μm respectively (data not shown). The separation distance D_L remains the same in both quality and speed modes at 160 μm . From SEM observation, the line width W appears to be larger than that of the ones printed in the quality mode (see Figure 3.19). In fact, it can be seen from the SEM image that the lines printed in speed mode have coalesced with one another. This is to be expected since the theoretical line width W is calculated to be 182.4 μm in the speed mode, which is larger than the separation distance D_L . Hence,

Figure 3.40: A) lines printed in quality mode, B) lines printed in speed mode

the alternate lines printed during the forward pass will join with one another before the printhead makes its return pass. Together, the lines printed in both the forward and return pass will result in a thicker layer, and hence a lower z resolution.

Base on mass conservation, the layer thickness, h_t , can be expressed as (see Figure 3.20 for illustration):

$$D_L h_t L = AL + A_{gap} L \quad (\text{Equation 3.20})$$

where A_{gap} is the cross sectional area of the gap that must be filled with the line printed in the returning pass. For a specific length L , since the volume of a line printed in the forward pass, AL , is the same as the volume of the line deposited in the returning pass, the volume of the gap can be expressed as:

$$A_{gap} L = AL$$

Equation 3.20 expressed per unit length is:

$$D_L h_t = 2A \quad (\text{Equation 3.21})$$

Figure 3.41: Geometrical analysis of layer thickness

Rearranging, we obtain layer thickness, h_t , as:

$$h_t = \frac{2A}{D_L} \quad (\text{Equation 3.22})$$

Substituting equation 3.19 into 3.22, we obtained the following:

$$h_t = \frac{\pi t^3}{3D_L} \left(\frac{f}{S} \right) \quad (\text{Equation 3.23})$$

Thus, the overall z resolution of the Objet printer is dependent on several parameters: the droplet frequency and printhead velocity, which can be set depending on the print mode chosen by the end-user, the droplet volume and also the separation distance D_L . The theoretical layer thickness calculated using Equation 3.23 is 2.96 μm and 13.08 μm for the quality and speed modes respectively. The difference from the values measured using the profilometer can be attributed to several factors. First, the volumetric expansion of the photopolymer during the curing process is not considered in the theoretical analysis. Secondly, there is presence of air bubbles in the layer, especially at the regions where the lines printed in the return passes interface with the ones printed in the forward pass. These air bubbles could have resulted in a larger layer thickness. Lastly, the estimated value for droplet velocity, U may be too far from the actual value. These collective factors may have brought about lower predicted values for the layer thickness.

3.2.7 Section Summary

Despite the lower predicted values for layer thickness, the theoretical analysis and the experimental studies described in this section have highlighted the differences between the two printing modes. Based on the droplet spreading model, the settings in quality mode (slower droplet frequency and a faster printhead speed) resulted in a smaller line width and hence, a smaller layer thickness as compared to speed mode. Thus, the quality printing mode possesses a higher build resolution. On the other hand, the speed mode, as its name implies, sacrifices quality for speed, allowing parts to be built at a faster pace due to the larger line width and the resulting larger layer thickness. In the inverse scaffold fabrication method, the mold is required to have

interconnected micron sized-struts which will eventually form the channels of the scaffold upon mold dissolution. Such micron-sized features require a high resolution to accurately reproduce the dimensions from CAD data. As such, the quality mode will be used in the experimental studies in the following section and also to print the RP component.

3.3 Dimensional Accuracy

According to the manufacturer's specifications, the accuracy of the Objet printer is within $\pm 100 \mu\text{m}$. For large models, the discrepancy between the dimensions of the fabricated part from their CAD design seems trivial. For example, an actual printed part with a diameter of 100.1 mm based on a CAD design of 100 mm diameter may not seem significantly different. For small micron-sized parts however, this difference becomes obvious. For example, a design of 400 μm diameter part and an actual printed diameter of 500 μm . Thus, the dimensions of the model may not be fabricated according to the CAD design especially for very small parts or parts with micron-sized features.

To investigate the feasibility of printing micron-sized features using Objet, parts were fabricated and dimensions were measured and compared with CAD data. The parts to be fabricated are strips with protruding rectangular features (see Figure 3.21). These features are to be printed in varied dimensions ranging from 150 μm to 1000 μm (in 50 μm increments). Recently, it was reported that the mechanical properties of parts printed using Objet changes according the orientation in which the part was printed . This is due to the printhead motion which is always traversing in the x - direction. Thus, it becomes necessary to study the dimensions of the parts fabricated in different orientations. Prior to fabrication, the CAD models were oriented in such a way that the rectangular features are aligned pointing in the x -, y -, x - y and y - x axis using the proprietary software, Objet Studio® (see Figure 3.22). Six samples were printed for each orientation and the width of the rectangular features were measured and tabulated in Table 3.2.

Figure 3.42: Part to be printed for measurement

Figure 3.43: Layout of the printed parts for measurement

Table 3.2: Summary of part measurements

Design width (μm)	X axis (μm)		Y axis (μm)		X-Y axis (μm)		X-Y axis (μm)	
	Mean	Std dev	Mean	Std dev	Mean	Std dev	Mean	Std dev
1000	963	10	1076	13	1030	14	1022	16
950	949	9	1033	25	956	5	953	7
900	883	10	952	13	908	10	915	12
850	840	19	914	31	896	19	911	13
800	801	11	858	18	829	17	823	12
750	708	10	789	33	787	16	788	21
700	656	17	777	21	729	13	735	11
650	613	11	729	16	670	10	672	8
600	572	23	681	11	630	12	619	14
550	532	14	651	16	606	9	602	11
500	570	19	562	10	591	13	597	11
450	532	12	554	15	587	5	590	8
400	508	18	488	7	584	7	575	12
350	533	10	476	11	583	9	581	10
300	490	19	468	10	530	14	541	19
250	442	23	427	14	461	12	457	11
200	353	20	410	11	368	18	354	25
150	-	-	-	-	366	18	-	-
100	-	-	-	-	-	-	-	-
50	-	-	-	-	-	-	-	-

Figure 3.23 shows the SEM images of the printed part that was designed at 550 μm . It should be noted that measurements of 150 μm and below are not attempted as the width is very small. From handling, the thin rectangular feature is too flimsy and unsuitable for measurement. Thus, the smallest printable feature that allows handling while at the same time retains its original shape is 200 μm and above. The data are plotted into a graph shown in Figure 3.24 (see Appendix A for larger image). It can be observed that at all orientations; the features that were designed at less than 500 μm width had a larger printed width. With the exception of parts that were aligned in the Y-direction, the width of all printed parts gradually becomes more accurate at widths larger than 550 μm . For y- direction, the fabricated width appears to be at least 50 to 100 μm larger than the corresponding designed value at all widths larger than 550 μm . Parts aligned in the x-direction are also observed to conform closest to the designed dimension at 800 μm and above.

Figure 3.44: Top view of the fabricated strip (oriented in the x-direction) showing the rectangular feature that was designed at 550 μm .

Figure 3.45: Plots of measured widths in various orientations. A) x- direction, B) y- direction, C) x-y direction, D) y-x direction

Comparing x- and y- direction, the measured values were significantly different from one another at all designed widths above 550 μm . In other words, any parts that were

fabricated in the *x*- orientation will have a slightly different dimension when fabricated in the *y*-orientation. For parts oriented in *x-y* and *y-x* directions, the measured values are not significantly different from one another. Therefore, in order to fabricate scaffold with interconnected channels in the *x*, *y* and *z* axis and to ensure uniform channel diameters, the RP mold should be oriented in a diagonal orientation (in the *x-y* axis) during preparation of the CAD data via Objet Studio®. In addition, the channel diameter should be set at sizes of 550 μm or above so that the fabricated channel diameter is not too far from the designed value.

3.4 Chapter Summary

This chapter has discussed the fundamentals underlying the PolyJet™ printing process with some experimental work. The Objet (Eden 350v) printer operates using micron-sized photopolymer droplets as its basic building units in the fabrication of a 3D part. The droplet jetted from the printhead nozzle and its subsequent spreading behavior would define the resolution of the process. Based on the droplet spreading model, the line formation process and the analysis of the layer thickness in the two print modes were discussed. In contrast to the speed mode which sacrifices resolution for a faster build time, the quality mode allows a high resolution printing process due to the smaller line width and layer thickness. In addition, the feasibility of printing micron-sized features using Objet has been investigated. To fabricate scaffolds with channel diameters according to the CAD data, the RP mold design should consist of struts that are 550 μm or above. The RP mold should also be printed in the *x-y* orientation in order to ensure uniform channel sizes in both *x*- and *y*-axis. To produce the inverse mold, the RP mold will be filled with the second component, which is the microparticles and will be discussed in detail in the next chapter.

Chapter 4 – INVERSE SCAFFOLD FABRICATION PART II: MICROSPHERE PREPARATION

In the proposed inverse scaffold fabrication approach, the mold captures the negative replica of the entire design of the scaffold architecture. In order to achieve this function, the mold must not only contain struts to create the macro-channels in the scaffold, but also consist of components that will form the pores of the scaffold. As such, the RP mold alone will not be able to meet these requirements. As discussed in Chapter 2, the particulate leaching process often involves the use of micron-size particles that will eventually be leached to form the scaffold pores. For this research, this concept is applied by using these micro-particles as filler materials for the RP mold. Ultimately, these particles will fill up the entire RP mold in which the final combined structure translates to the entire scaffold structure.

This chapter presents the rationale for the choice of these particles, their production process and the fabrication method of the final mold. For simplicity, this final mold will be known as the inverse mold throughout this chapter. A novel technique that has been developed to arrange these micro-particles in the inverse mold will also be presented.

4.1 Requirements for the Micro-Particles

One of the motivations for the proposed inverse scaffold fabrication approach, in addition to overcoming the resolution limit of RP techniques, is to impart sufficient structural controllability to end-users. To achieve this goal, the choice of these particles, which are the building blocks for the scaffold pores, must coincide with certain criteria in order to successfully fabricate scaffolds with customized/controllable pore architecture. These are outlined in the following sections.

4.1.1 Solvent and Scaffold Material Compatibility

The solvent used to leach out the particles must not dissolve the scaffold material. This would require careful consideration of the particle/scaffold material combination to be employed in this fabrication process. For example, salt crystals cannot be used in conjunction with water soluble scaffold materials since the water used to leach out the salt crystals will also dissolve the scaffold material at the same time. In short, the hydrophobic/hydrophilic criterion must be fulfilled. If hydrophobic particles are used, the scaffold material must be hydrophilic (and *vice versa*) since the solvent used to dissolve the particles will be hydrophobic as well. As discussed in Chapter 2, gelatin, a hydrophilic material will be used as the scaffold material. Thus, the choice of particles for this research will be of hydrophobic nature.

4.1.2 Uniform Particle Shape and Size

As discussed in Chapter 2, irregular-shaped particles tend to compromise pore interconnection within the scaffold. To achieve a highly interconnected pore network, particles of similar size and regular shape (mono-dispersed) could be used so as to maximize the contact between individual particles.

4.1.3 Ease of Production and Availability in Different Sizes

The particles should be easy to mass-produce without the need for any specialized equipments. As different pore sizes may be required for different scaffolds, these particles must also be able to be produced into a range of different sizes.

Based on the criteria discussed above, microspheres are suitable candidates compared to other forms of particles such as salt and sugar crystals as they are completely spherical in shape. Details concerning the fabrication of microspheres are well-documented. As such, microspheres will be used as filler materials for the RP mold in this work. As gelatin is the proposed scaffold material for this research, the scope of microsphere materials is narrowed down to hydrophobic materials e.g., Poly (methyl methacrylate) (PMMA), polystyrene, PCL, PLA etc.

4.2 Inverse Mold Fabrication Outline

The various stages involved in the fabrication of the inverse mold are shown in Figure 4.1. The microspheres, after fabrication, are to be separated according to their sizes and stored for later use. These microspheres are to be packed into the RP mold using an ultrasound agitation method. This method allows the microspheres to be packed in an ordered arrangement and will be discussed in detail in a later section. Thereafter, the microspheres and the RP mold are fused together by means of sintering. This is to fix the microspheres in place as well as to form permanent contacts between the microspheres, a process called necking. These permanent necks will ultimately form the interconnection between individual scaffold pores.

Figure 4.46: The various stages of inverse mold fabrication

4.3 Microsphere Preparation

4.3.1 Microsphere Material

The choice of microsphere material is an important consideration since different materials will have different properties which may have implications on the whole fabrication process. During the sintering process, the microspheres partially melt and

fuse with one another, forming necks between every contacting microspheres. To preserve the structure of the RP mold during the sintering process, the glass transition temperature of the selected material should be lower than the melting point of the RP mold at 115 °C. In this case, polystyrene was chosen as the microsphere material as it has a transition temperature of 95°C, above which it becomes ‘sticky’, allowing the microspheres to fuse together.

4.3.2 Microsphere Fabrication

The oil-in-water emulsion technique is a relatively simple microsphere fabrication method and allows microspheres to be produced in bulk quantities without the need for specialized equipments. Basically, the microsphere material is dissolved in a solvent and poured into a beaker of water, forming a ‘oil in water’ suspension. The suspension is subjected to agitation to break up the ‘oil’ into small droplets. Due to agitation, the solvent evaporates over time and the droplets solidify, forming the microspheres.

1.5g of polystyrene (Sigma-Aldrich molecular weight 192000) was dissolved in 20ml dichloromethane (DCM). The dissolved polystyrene was added into 400ml of deionized (DI) water containing 0.5% of polyvinyl alcohol (PVA). Note that the addition of PVA in DI water is an important step as it functions as a surfactant and prevents the dispersed droplets from coagulating. The emulsion obtained was then mechanically stirred for five hours using a simple setup (see Figure 4.2) to disperse the droplets and to evaporate the DCM.

Figure 4.47: Polystyrene microsphere fabrication using oil-in-water emulsion technique

4.3.3 Separation and Storage

As discussed in chapter 2, using uniform shaped particles with a narrow size distribution would help to maximize packing and consequently, pore interconnectivity. Microspheres produced using the oil-in-water emulsion technique often have a wide range of sizes. Similarly, the polystyrene microspheres fabricated in this work have a large size distribution which needs to be separated according to their sizes (see Figure 4.3).

Figure 4.48: Polystyrene microspheres of random sizes (20X magnification, scale bar: 100 μm)

4.3.3.1 Microsphere Separation

The microspheres were separated into a much narrower size distribution using standard sieves. Microspheres of sizes smaller than the mesh size of the sieve will pass through the mesh to the next sieve whereas those with larger sizes will be trapped (see Figure 4.4). The sieves were stacked according to descending mesh sizes: 163 μm , 155 μm , 112 μm , 106 μm , 100 μm , 95 μm , 90 μm , 85 μm , 63 μm , 60 μm , 56 μm and finally 53 μm . The beaker containing the solution of polystyrene microspheres was immediately poured onto the top sieve, allowing the PVA solution as well as microspheres smaller than the required mesh sizes to drain off from the bottom sieve.

Figure 4.49: The sieving process

Due on the abundance of polystyrene microspheres that were collected in the mesh size range of 85 to 90 μm (average size of 88 μm) (see Figure 4.5), they will be mainly used for scaffold fabrication in the following chapters. To prevent aggregation of the microspheres, the top sieve was continuously rinsed with DI water and sprayed with 100% isopropanol (IPA) to disperse the microspheres. The use of IPA will be an important aspect of the scaffold fabrication process and will be discussed in the next section.

Figure 4.50: Polystyrene microspheres with an average diameter of 88 μm

4.3.3.2 Handling and Storage

After the sieving process, the separated microspheres were transferred to a 100 ml beaker. For these microspheres to be viable as ‘building blocks’ for the scaffold, they must be able to exist as single, individual units and not as clumps which will be difficult to assemble in the RP mold. Therefore, the choice of storage medium is an important consideration. Water, being a hydrophilic medium will be not effective in dispersing the microspheres as large aggregates can still be seen (see Figure 4.6). In a colloidal suspension consisting of hydrophobic particles in a hydrophilic medium, the particles tend to aggregate around one another. There are several factors that will cause particles to aggregate:

- Particle size - The likelihood of aggregation increases as particle size decreases. Brownian motion of smaller particles are more likely to cause hydrophobic interactions among particles.
- Particle concentration – As the concentration of the microspheres increases, so does the likelihood of collisions among individual microspheres. This increases hydrophobic interactions among the particles and eventually leads to aggregation.
- Surface charge – Similar charged particles causes repulsion between one another, preventing aggregation. The lower the amount of charge, the lower the colloidal stability in the solution. This increases hydrophobic interactions and the tendency to aggregate.

Figure 4.51: 53 μm polystyrene microspheres in water with aggregation (indicated by the circles)

The interaction between surfaces is a widely studied area in the field of colloidal science. In a hydrophilic system (2 hydrophilic particles), a long range repulsive force exists between their surfaces in water. Polystyrene particles generally have highly hydrophobic surfaces. In the case of a hydrophobic system (2 hydrophobic surfaces), a long range attractive force (the hydrophobic force) exists between the particles. In a suspension of water with hydrophobic particles, the attraction force dominates over other inter-particle forces e.g., van der Waals forces. Thus, the collisions and random Brownian motion between particles can cause the particles to aggregate. Researchers have experimented with the addition of alcohols in colloidal suspensions to reduce the attractive forces between hydrophobic surfaces. In Hupka's work, the use of IPA has been demonstrated to diminish the attractive forces in the case of a hydrophobic system. Similarly, 100% IPA was used as the storage medium for this research. Figure 4.7 shows the microscopic image of mono-dispersed polystyrene microspheres of 53 μm in IPA. They appeared to be fully dispersed in the liquid phase with no signs of aggregation.

Figure 4.52: 53 μm polystyrene microspheres in IPA

4.4 Microsphere Packing

For the scaffold to achieve maximum porosity, the microspheres must be arranged into their closest packed arrangement. In this manner, high interconnectivity is also ensured as the majority of the microspheres will achieve contact with one another. Figure 4.8 illustrates the ideal scenario of having equal sized-microspheres stacked together in a hexagonal closest packed (HCP) arrangement. Here, every individual microsphere is in contact with at least 12 neighboring microspheres. In such an arrangement, the number of contact between microspheres is at its maximum, which means that a network of highly interconnected pores is ensured.

4.4.1 Method of Assembly

In the field of colloid science, there has been considerable interest in the self-assembly of nano-sized spheres into colloidal crystals. In the assembly of the atoms, molecules and nanoparticles that form colloidal crystals, thermal energy provides the kinetics; the resultant motion e.g., Brownian motion allows particles to attain the optimum positions to create highly ordered structures. Due to the size of nanoparticles, the large surface area maximizes inter-particle forces e.g., van der Waals forces, electrostatic forces, capillary forces, surface tension etc. which further enhance the self assembly process.

Figure 4.53: Microspheres in Hexagonal closed packed arrangement and resulting scaffold

When a drop of a suspension of nanospheres is allowed to dry on a substrate, the receding three phase contact line of the droplet at the liquid, substrate, and air interface and the capillary forces driven by the evaporation of the suspending liquid will result in the accumulation of spheres, self-assembling into a blend of ordered and disordered regions. During the process, the evaporation of the liquid creates a meniscus between the particles and induced a capillary action which attracts the particles together (see Figure 4.9). This meniscus is only formed when the thickness of the liquid film drops below the particle size. As the evaporation proceeds further, the particles enclosed within the droplet are gradually packed together until the droplet completely evaporates, resulting in a closed packed structure.

Figure 4.54: The evaporation-induced self assembly process

However, evaporation induced self assembly is only applicable to very small particle sizes. There are other external forces which come into play and are largely influenced by the particle size. As the particle size increases, external forces such as gravity, hydrodynamic drag and frictional forces will dominate over colloidal forces. The latter is more significant for small colloidal crystals. For particle sizes of more than $1\ \mu\text{m}$, thermal motion will be insufficient in providing the kinetics for self assembly. Gravitational energy plays a larger role and the kinetic energy required to move the particles increases due to the larger particle mass. Furthermore, the increase in friction forces makes it more complicated for self assembly: friction force is approximately proportional to the particle weight and thus, proportional to the third power of their size. Therefore, the additional kinetic energy required for the assembly of larger particles must be supplied by other means, such as agitation or pumping.

In tissue engineering applications, the pore sizes of scaffolds are generally in the range of tens to hundreds of microns. The polystyrene microspheres fabricated in this research falls into this size range. To achieve an assembled structure of polystyrene microspheres, the evaporation-induced self assembly process was first attempted in this study. Briefly, a 10 μl droplet of IPA containing 100 μm polystyrene microspheres was dispensed onto a glass slide and left to dry. Figure 4.10 shows the microscopic arrangement of microspheres on the glass slide after complete evaporation of the IPA. Clearly, the capillary forces induced by evaporation alone were insufficient in producing an ordered, close packed assembly of microspheres.

To maximize the packing efficiency of the polystyrene microspheres, the evaporating IPA was subjected to agitation. Two methods were explored in this study:

Method 1 - A 10 μl droplet of IPA containing 100 μm polystyrene microspheres was dispensed onto a glass slide and attached atop an orbital shaker (Thermolyne Roto Mix Type 50800) (see figure 4.11A). The shaker was rotated at varying speeds from 80 rotations per second (RPM) to 200 RPM to induce agitation to the microsphere suspension while the IPA evaporates.

Figure 4.55: Arrangement of 100 μm polystyrene microspheres after evaporation of IPA with no agitation (scale: 500 μm)

Figure 4.56: Methods of providing agitation to microsphere suspension to induce assembly: A) orbital shaker method, B) ultrasound vibration method

Method 2 – This method employs ultrasound to agitate the microsphere suspension (see Figure 4.11B). A glass slide containing $10\ \mu\text{l}$ of $100\ \mu\text{m}$ microspheres suspension was placed in a holder in an ultrasonic bath such that the water level is just in contact with the bottom of the glass slide. While the suspension was subjected to ultrasound vibration, the assembly process was visualized using a high speed camera.

The microscopic image of the assembled microspheres using the orbital shaker method (method 1) is shown in Figure 4.12. From the figure, it can be observed that the assembled microspheres consist of both ordered and disordered regions. The variation in rotation speed did not produce any noticeable improvements in the arrangement of the microspheres (figure not shown). Note that for this method, it was not possible to capture the assembly process of the microspheres using a high speed camera due to the large horizontal displacement of the rotating platform and the small focus size of the high speed camera.

Figure 4.57: Arrangement of 100 μm polystyrene microspheres in IPA using the orbital shaker method (scale bar: 200 μm)

On the contrary, ultrasound agitation was able to produce an ordered, HCP array of polystyrene microspheres. The images captured from the high speed camera during the assembly process are shown in Figure 4.13. Clearly, the vibration on the glass slide was able to move individual microspheres into their closest packed arrangement. Pouliquen *et al* suggested that the relative acceleration of the vibration, $\Gamma = A\omega^2 / g$ affects the packing behavior of spheres, where A is the vibration amplitude, ω is the frequency of vibration and g is the gravitational acceleration. In their study, they observed that a low relative acceleration is ineffective in producing an ordered, closed packed microsphere assembly. The vibration provided by the ultrasonic bath has a very high frequency and hence a large relative acceleration that leads to an ordered alignment of the 100 μm polystyrene microspheres. From the video captured by the high speed camera, the microspheres can be seen shifting about the glass surface in small displacements while still enclosed in the IPA film. As the IPA evaporates, the liquid film becomes smaller, and the contact line starts receding. The enclosing IPA film then forces the microspheres to pack together. In normal cases, this would not be possible due to the large microsphere size (Figure 4.10). It is believed that the vibration induced by the ultrasound causes the microspheres to periodically ‘jump’ and lift off the glass surface in very small displacements and at high frequency. While the microspheres are not in contact with the glass surface, the enclosing IPA film

begins to push the microspheres towards the center of the film, while keeping them enclosed in the IPA suspension. This process continues until the microspheres cannot be packed further and the IPA completely evaporates.

Figure 4.58: Arrangement of 100 μm polystyrene microspheres in IPA subjected to ultrasound agitation

4.4.2 Stacking of Microsphere Layers

The ultrasound assembly method was investigated using a larger volume of microsphere suspension. Briefly, a 50 μ l suspension was dispensed onto a larger glass plate and placed atop the ultrasonic bath holder. As with previous experiment, the assembly process was captured via a high speed camera. Interestingly, the vibration on the glass plate was able to induce stacking of the microspheres into several layers (see Figure 4.14). The assembly process of the first layer was somewhat similar to that of Figure 4.13. After the first layer of assembled microspheres was formed, a small area of concentrated microspheres can be seen protruding above the first layer (see Figure 4.14D). This ‘seed’ layer is responsible for the formation of the second stack of microspheres. From figure 4.13E to 4.13L, this small area of concentrated microspheres began to propagate outwards as it ‘recruited’ the microspheres from the bottom until the second layer was complete. In the process, the area occupied by the first layer gradually became smaller. This is expected as the microspheres that previously resided in the space of the first layer now stayed at the second layer. As the stacking continued into the third layer and so on, the area became smaller.

Figure 4.59: Demonstration of self stacking during assembly of 100 μm polystyrene microspheres

The number of layers that can be stacked is associated with the thickness of the glass plate. As the thickness decreases, the number of stacking layers increases (see Figure 4.15). The experiment was conducted using glass slides stacked on top of one another (up to 3 glass slides) and placing it in the ultrasound bath. The change in layer thickness can be attributed due to the damping effect caused by increasing glass slide thickness. As more glass slides were stacked on top of one another, the vibrations were damped and the kinetic energy provided was unable to lift off the microspheres from the upper stacks, thus resulting in very few layers of assembled microspheres. Nonetheless, this self stacking phenomenon of microspheres is a feasible technique for fabricating thin large films of scaffold (up to 2mm) with an ordered, uniform array of customized pore sizes. The films can then be cut into sizes that are desired for the target application using a blade.

Figure 4.60: The effect of glass thickness on stacking thickness. A) 1, B) 2 and C) 3 glass slides

4.4.3 Microsphere Assembly in RP Mold

From the above-mentioned experiments, the use of ultrasound vibration is able to achieve an ordered array of close-packed microspheres and is able to self stack into multiple layers. As such, the assembly of microspheres in the RP mold was carried out using the ultrasound agitation method.

Instead of using the glass slide, the RP mold was placed onto the ultrasonic bath holder and situated at a level where the water is touching the bottom of the mold. Using a micro-pipette, 85 μm polystyrene microspheres were dispensed into the mold

in a drop-wise manner while it is being subjected to ultrasonic vibration (see Figure 4.16). The feeding rate of the microspheres into the mold can affect the degree of order in the arrangement of the microspheres. It was suggested that a high feeding rate will likely result in a disordered crystal structure that consists of stacking faults e.g., grain boundaries, uneven stacking etc. To investigate the effect of feeding rate on the 3D assembly of microspheres in the mold, we employed two different feeding rates: a) $50\ \mu\text{l}$ per drop dispensed at every 1 min interval and b) $200\ \mu\text{l}$ per drop dispensed at every 30 seconds interval. Images were then taken from the top section of the RP molds after the sintering process, which will be discussed next. After the assembly process, the mold was left to stand for 12 hours at ambient conditions to allow complete evaporation of the IPA.

Figure 4.61: Experimental setup for dispensing the microspheres into the RP mold

4.5 Mold Sintering

The final step in creating the inverse mold is the sintering process which involves subjecting the microsphere-filled mold to high temperatures. Following the evaporation of the IPA solvent, the mold was transferred to a vacuum oven and subjected to heat treatment at 110°C overnight. At this temperature, the microspheres partially melted and began to fuse together. During this process, fusion necks were created between the spheres, thus ensuring the connectivity between microspheres and setting the entire structure in place. Although it is a simple process, the fusion

between the microspheres is an essential aspect of the scaffold structure as the necks that are created correspond to the interconnectivity between individual scaffold pores.

The final inverse mold is shown in figure 4.17. From the images taken from the top section of the mold, the feeding rate of $200\ \mu\text{l}$ per 30 seconds resulted in a structure with obvious regions of misaligned microspheres (see Figure 4.17 B). On the contrary, the slower feeding rate of $50\ \mu\text{l}$ per minute was able to produce a much more ordered array of microspheres, although grain boundaries can still be seen (see Figure 4.16 C). This can be attributed to the consistency in the order of the first assembled layer. If the first layer is well assembled, then the following layers are likely to assume the same alignment. Figure 4.18 shows the upper portion of a 3D assembly of microspheres that is created using the same experimental method

Figure 4.62: A) The fabricated Inverse mold (comprising of the sintered RP mold and the microspheres), B) image of the assembly of microspheres using a feeding rate of $200\ \mu\text{l}$ per 30 seconds and, C) feeding rate of $50\ \mu\text{l}$ per minute

(feeding rate of 50 μl per minute with ultrasound agitation) on a glass slide enclosed with a circular Teflon ring. The top few layers appeared to be orderly arranged in a HCP configuration, suggesting that the bottom layers could be arranged in a HCP configuration as well. At a lower feeding rate, there was sufficient time for the first layer of the microspheres to form and align itself. The first layer of microspheres that assembled at the bottom is the most important layer since their rugged surface will become the template for the formation of the second layer, and so on. This would also mean that misalignments in the structure, if any, will accumulate from the bottom layers. As more microspheres were added, they aligned and positioned themselves according to the template. For high feeding rates, the disordered regions observed suggest that the first layer of microspheres had insufficient time to assemble itself. Therefore, the microspheres in the bottom layers were locked in their disordered arrangement as more microspheres were added and began to pile on top of one another. The misalignments created in the bottom layers continued to build up to the top layers and could not realign itself even with vibration.

Figure 4.63: A 3D view of the assembly of microspheres showing an ordered HCP alignment in every layer

4.6 Chapter Summary

In this chapter, the fabrication process of the inverse mold has been investigated and developed. The process involved the use of ultrasound agitation to assemble the polystyrene microspheres into their closest packed arrangement in the RP mold. The feasibility in producing an ordered 3D assembly of microspheres through the combination of ultrasound agitation, a suitable solvent (IPA) and a low microsphere feeding rate has also been verified. The fabricated inverse mold corresponds to the negative replica of the entire scaffold architecture: the microspheres ultimately form the pores of the scaffold, and the RP mold determines the macro features e.g., channels and the shape of the scaffold. By arranging the microspheres into their closest packed arrangement, scaffold porosity is maximized. Polystyrene microspheres with an average diameter of 88 μm were chosen for scaffold fabrication due to the abundant amount collected during the sieving process. It is to be noted that the selected size of 88 μm is not within the optimal range of pore sizes discussed in the literature review in chapter 2. The focus of this work is emphasized on developing the inverse fabrication method and laying out the guidelines of the fabrication procedures. Nonetheless, the use of other microsphere sizes in the inverse scaffold fabrication method is still applicable if given enough quantity. The next chapter discusses the modeling of the necking process and how they affect the interconnectivity between pores.

Chapter 5 – THEORETICAL ANALYSIS OF SCAFFOLD STRUCTURE

In the inverse scaffold fabrication approach, the structure of the inverse mold determines the structural characteristics of the scaffold. For example, the packing density of the microspheres would also correspond to the porosity of the resulting scaffold. For a scaffold fabrication technique to have a sufficient degree of structural control, users must be able to predict and control the key stages of the fabrication process. As such, models are necessary to simulate the process and/or predict the outcome of the scaffold structure. In this research, the size uniformity and the close packed, ordered arrangement of the microspheres suggest that the pore structure of the resulting scaffold can be characterized analytically. As discussed in chapter 4, the sintering stage in the fabrication process of the inverse mold partially melts the microspheres and fuses them together. Fusion necks are created at the point of contact between the microspheres and these necks correspond to the interconnectivity between the pores of the resulting scaffold. In this chapter, a theoretical analysis based on Frenkel's sintering model describing the parameters of the sintering process and its relation to the resulting characteristics of the scaffold structure is presented.

5.1 Microsphere Sintering Process

Sintering is a process usually defined as the formation of a homogeneous melt from the fusion of solid particles under high temperature. The process causes the particles to form bonds that are needed to hold the entire mass together. The fusion of polymer particles is often done at temperatures above their melting point for crystalline materials or above their glass transition temperature for amorphous materials. In this research, the objective of the sintering process is not to fully melt the polystyrene microspheres but rather, to achieve partial fusion. This allows the microspheres to retain their basic spherical shape while having spaces of gaps in between to allow for scaffold material infiltration during the casting process.

5.1.1 Phases of Sintering

Sintering occurs by atomic diffusion processes that are stimulated by high temperatures. The type of sintering mechanism will ultimately depend on the type of material being sintered. These mechanisms (e.g., volume diffusion, grain boundary diffusion, plastic flow and viscous flow etc.,) usually result in shrinkage or densification of the whole particle system. In general, amorphous materials sinter by viscous flow, which has been extensively studied by numerous researchers. On the other hand, crystalline materials can sinter by one or more mechanisms occurring separately or in parallel depending on the material system. The entire sintering process is generally categorized in 3 phases: a) initial stage, b) intermediate stage, c) and final stage (see Figure 5.1). There are no clear-cut distinctions between the phases as the process associated with each phase tends to overlap one another. Still, some generalizations can be made to distinguish one stage from the next. During the initial phase of sintering, the interface between the contacting particles, or microspheres in the case of this research, forms a polymeric bridge, or neck that grows in radius over time. Densification of the particle system begins at this phase. The formation of necks between particles can take place by diffusion, plastic flow or viscous flow. The next phase is assumed to begin when neck

Figure 5.64: The 3 phases of the sintering process

radii reaches around 0.4 to 0.5 times of the particle diameter. In this phase, the gaps between the particles become more defined and form individual ‘pores’ that begin to isolate from one another. This phase covers the majority of the whole sintering process. The final phase encompasses the closing up of the pores until the particle system is completely densified.

5.1.2 Maximum Neck Length

The maximum possible neck length that can be achieved through the use of microspheres in this research can be illustrated by considering a two dimensional (2D) array of spheres that are aligned in a HCP arrangement. Before the initial phase, the microspheres are assumed to be just touching each other with zero neck length (see Figure 5.2a). For a hexagonal 2D array of microspheres, a maximum neck length of approximately half a microsphere diameter, r is achieved at the end of the sintering process (see Figure 5.2b).

Figure 5.65: Illustration of neck length A) before sintering and, B) after the final phase of sintering

In a 3D, HCP assembly of microspheres, there will still be gaps in between individual microspheres at this maximum neck length of $0.5d$ (see Figure 5.3). However, necking beyond this maximum neck length is not critical in this study because the scaffold material will not be able to infiltrate into the mold through the first 2D layer of microspheres during the casting process. Thus, the initial phase of the sintering

process is of interest and neck growth beyond this point will not be explored in this research. In this analysis of neck length at maximum necking (neck length = $0.5d$), it is assumed that the scaffold material deposits at the surface around the neck region, which is represented by the blue surfaces shown in Figure 5.3. Eventually, the red regions form the interconnection between the scaffold pores when the inverse mold is dissolved.

Figure 5.66: Alignment of microspheres at maximum necking length in a 3D view

5.1.3 Driving Forces of Necking

The necking process begins when the particle system is raised to a temperature high enough to enable material transport processes to occur at relatively fast rates (relative to room temperature). Surfaces and interfaces have associated energies (e.g., surface tension) that vary as a function of surface curvature. The driving forces for these transport mechanisms are characterized by the tendencies of the system to reduce its total free energy. This is accomplished by material transport from regions of low curvature to regions of high curvature. Thus, by reducing or eliminating these regions of high curvature, the overall energy of the material system is reduced. The numerical simulation from the work of Djohari *et al* is used to illustrate an example. When two microspheres are brought into contact, a small neck region of high curvature is formed (see Figure 5.4). At this region, the surface tension effects are much higher than on the individual microsphere surfaces and this creates the driving force for material transport. As sintering proceeds, material flows are driven towards the neck area where the neck radius increases along with a decrease in surface curvature over time.

This is also accompanied by a decrease in overall system length (the size of the material system) leading to densification which corresponds to an increase in packing density. Thus, the entire system tends towards a system of minimum surface area eventually leading to a single sphere after the final phase of the sintering process.

Figure 5.67: Numerical simulation of the necking process of 2 spheres showing the increase in neck radius along with the decrease in surface curvature at the neck region and the eventual decrease in total surface area of the material system over time

5.2 Theoretical Analysis of Pore Interconnection Size and Scaffold Porosity

The first sintering model describing the necking process of two equal sized spheres was developed by Frenkel *et al* in 1945. . The proposed model, which is based on Newtonian viscous flow under the action of surface tension, has frequently been used to illustrate the kinetics of viscous sintering. Despite its simplicity, the model gives an insight of the effect of material properties and particle size on the necking process. Several other models have since been developed to modify Frenkel's model accounting for various sintering systems e.g., particles of irregular sizes, different materials etc. These models can be written in a general form :

$$y = kr^a t^b \quad (\text{Equation 5.1})$$

where y is the neck radius, k is a constant which depends on material properties such as surface tension, viscosity, modulus etc. r is the microsphere radius and t is time. The parameters, a and b are functions of the transport mechanisms, as shown in Table 5.1. For Newtonian viscous flow, the neck grows according to:

$$\frac{y}{r} = k \sqrt{\frac{t}{r}} \quad (\text{Equation 5.2})$$

Taking the time derivative of Equation 5.2:

$$\frac{d(y/r)}{dt} = \frac{k}{2} \sqrt{\frac{1}{rt}} \quad (\text{Equation 5.3})$$

Equation 5.3 illustrates 2 important aspects of the necking process. Firstly, the rate of growth of the neck ratio $\frac{d(y/r)}{dt}$ is inversely proportional to the square root of the microsphere radius. As such, smaller particles tend to have a faster rate of neck growth. Secondly, the rate of growth of neck ratio is also inversely proportional to the square root of time. Thus, the rate of neck growth decreases with time and it takes place most rapidly at the early phases of sintering.

Table 5.3: Scaffolds and approaches

Sintering mechanism	a	b
Viscous flow	1/2	1/2
Evaporation/condensation	1/3	1/3
Surface diffusion	3/7	1/7
Bulk diffusion	2/5	1/5
Elastic deformation	2/3	0

5.2.1 Neck Radius

Frenkel's model is limited to the early stages of sintering, which is of concern in this research. In his model, the change in neck radius, y with time, t is given as :

$$y^2 = \left(\frac{3r\gamma t}{2\eta} \right) \quad (\text{Equation 5.4})$$

where η and γ are the viscosity and surface tension of the microsphere material respectively. This model is derived by balancing the work of surface tension and viscous dissipation in the sintering system. Frenkel assumed the sphere radius to be constant throughout the process. In actual sintering systems however, particle radius changes with time and the applicability of Frenkel's model in the later stages of sintering might not be appropriate. There were models that have been developed to take into account for the change in particle size over time . From preliminary investigations however, the size of the polystyrene microspheres (88 μm diameter) after the sintering process didn't not appear to change significantly (see figure 5.5). Furthermore, experiments with polystyrene microspheres has been reported to agree well with Frenkel's predictions . Thus, the Frenkel's sintering model will be used to predict the neck radius of the sintered microspheres in this work. The size of the neck will ultimately correspond to the size of the interconnection that links the scaffold pores.

Figure 5.68: Sintering of 88 μm polystyrene microspheres for 32 hours

Before the onset of sintering at time, $t = 0$, two similar sized spheres both with radius r are initially in contact at point O. As sintering progresses, the center of origins of the two spheres move towards the point O, forming an intersection at the necking area (see figure 5.6). The angle of the intersection and the neck radius at time t is θ and y respectively. In the sintering process, users are able to control two parameters: the sintering time interval and the sintering temperature. From Equation 5.4, the change in neck radius as a result of sintering time is straightforward: a longer sintering time interval would result in a larger neck radius. On the other hand, neck radius is inversely proportional to the viscosity η of the microspheres, which is in turn, dependent on the sintering temperature. A higher temperature would result in a lower viscosity, and hence a higher neck radius. However, the effect of sintering temperature on neck radius is not investigated in this study due to the limited availability of polystyrene microspheres. Furthermore, investigating the effect of sintering time on neck radius would be more straightforward as the other method requires an additional step of obtaining a viscosity value which destroys the microsphere sample and a new sample must be made to measure the neck radius.

Figure 5.69: The necking of two spheres at $t = 0$ and $t = t$

5.2.2 Local Porosity

The importance of having a scaffold with high porosity was discussed in chapter 2: to maximize space for cell adhesion and migration. Very often, controlling the porosity also affects the mechanical strength of the scaffold structure and this brings about conflicting design requirements as well. Thus, the evaluation of the scaffold structural parameters is necessary as it facilitates the tissue engineer in the improvement of the scaffold design process and its eventual applications. In this section, the porosity of the scaffolds fabricated via the inverse fabrication method is of interest. There have been several methods devised by various research groups to estimate scaffold porosity. In general, these methods can be classified into two main concepts: mass analysis and unit cube analysis. Mass analysis has been adopted by most researchers due to their simplicity which requires only the density, volume and dimensions of the scaffold in most cases. With this information, the scaffold porosity can be derived using this general formula:

$$Porosity = \left[1 - \left(\frac{V_f}{V_a} \right) \right] \times 100\%$$

in which V_f is the volume of the scaffold material and V_a is the apparent volume of the scaffold which is calculated from the dimensions of the scaffold ($l \times b \times h$). In general, the mass technique can be used for scaffolds with non-homogenous pore structures. One of the main limitations of this technique is that the accuracy of the porosity prediction is dependant upon the precise measurement of the scaffold dimensions (length, breadth, height). Therefore, edge effects introduced during the process of fabricating the scaffold is of concern as rough edges are created which can result in inaccurate dimensions and wrong porosity values. Other variations of the mass technique include the Archimedes method and the liquid displacement method which does not require the dimensions of the scaffold in the analysis. However, they would still require the mass or volume of the scaffold to be measured prior to obtaining the porosity value. The unit cube analysis is generally adopted for scaffolds with structures which follows a highly regular/periodic pattern e.g., scaffolds fabricated via RP techniques. In this approach, each repeating pattern within the scaffold structure is assumed as a unit cube and the porosity is derived by analyzing the volume of space and scaffold material within that cube. Khalil *et al* used the unit

cube approach to study the porosity of alginate scaffolds fabricated via an in-house developed biopolymer deposition system. In his analysis, the flow rate of the biopolymer is of concern as it affects the diameter of the deposited struts which are aligned parallel to each other in every layer. Thus, the porosity of the resulting alginate scaffolds is dependent upon the biopolymer flow rate adopted in the fabrication process. Some research groups have also adopted the unit cube analysis to derive the porosity of FDM fabricated scaffolds. In their analysis, it was assumed that the struts are of uniform diameter and each layer of struts formed does not fuse into one another. Although these assumptions simplify matters, it may contribute to errors as strut diameters tend to be inconsistent due to the flow dynamics of the extruded polymer and there exist partial fusion between the struts in actual FDM-fabricated scaffolds. For this research, this problem is minimal as the scaffolds do not consist of struts that are partially fused together as in the case of FDM scaffolds. In addition, the use of microspheres which are aligned in a regular HCP arrangement suggest that the porosity can be derived based on unit cube analysis. Thus, the unit cube approach will be adopted in deriving the porosity of the scaffolds fabricated using the inverse fabrication approach.

In this research, the term local porosity encompasses the regions of the scaffold that are bounded between the channels (see Figure 5.7). This is to differentiate the regional porosity from the overall (global) porosity of the scaffold which takes into account the space occupied by the interconnected channel network. During the sintering process, the gaps between the microspheres will gradually become smaller and eventually close up over time. This corresponds to an increase in packing density as neck radius increases. With the assumption of a perfect HCP microsphere structure, the packing density, which also corresponds to the scaffold porosity, is at 0.74. Thus, any further sintering, because of the ‘inverse’ fabrication process, would increase the packing density, and thus scaffold porosity above 0.74.

Figure 5.70: Illustration of local porosity of a scaffold

5.2.2.1 Unit Cell Concept

To predict the packing density of the microspheres at sintering time t , we assign a unit cell for every individual microsphere. If each microsphere is considered to be confined within its unit cell, and under the assumption of a perfect HCP structure, each unit cell will be of equal volume and the packing density of the microspheres can be calculated from a single unit cell: by dividing the volume of a single microsphere by the volume of the unit cell. Figure 5.8 illustrates a hexagonal unit cell in 2D. In the analysis of the unit cell volume for a 3D assembly of microspheres, each layer of spheres within the HCP assembled structure is divided into 2 regions – a region occupied by portion of spheres from only within the same layer, and another region where two layers of spheres overlap each other, as shown in Figure 5.9b. The height, y_1 , and height of the overlapping region, H_o , can be determined by analyzing the geometry of a regular tetrahedron, which is formed by joining the centers of four neighboring spheres with straight lines as shown in Figure 5.10.

Figure 5.71: 2D illustration of a unit cell (hexagon)

Figure 5.72: Analysis of a 3D HCP structure A) 3D isometric view, B) Side view of the two regions of a single microsphere layer

Figure 5.73: A) 3D illustration of a regular tetrahedron, B) dimensions of the tetrahedron

Each vertex of the tetrahedron represents the center of the four spheres and each side of the tetrahedron, l_{cc} represents the distance between the center of one sphere and the center of the neighboring contacting microsphere. This distance is denoted by l_{cc} (center-to-center length). l_{cc} is dependent on the extent of necking of the microspheres. From figure 5.10b, h is the vertical line starting from the top vertex of the tetrahedron and ending at the midpoint of the triangular base. It is represented by $h = l_{cc} \sin(54.74^\circ)$. At time $t = 0$ where neck radius is zero, ie. the spheres are just touching each other; l_{cc} is two times the radius of the microsphere. After a period of time t , the center-to-center length, l_{cc} decreases and can be calculated from the angle of intersection and the neck radius (see also Figure 5.6):

$$l_{cc} = \frac{r}{\tan \theta} \quad (\text{Equation 5.5})$$

Thus, as l_{cc} changes over time, the unit cell volume is expected to change too (see Figure 5.11). Considering the geometry of 2 microspheres after a period of sintering (see Figure 5.12), the height of a spherical cap h_c is given by:

$$h_c = r - \frac{r}{\tan \theta} \quad (\text{Equation 5.6})$$

Figure 5.74: Illustration of the change in unit cell over time

Figure 5.75: Geometry of 2 necked microspheres

The height y_1 and H_o can be determined by considering the shaded plane shown in the tetrahedron in Figure 5.10, which is further illustrated in Figure 5.13. The following equations can be derived:

$$x_1 = r \tan (\varphi - \alpha) \quad (\text{Equation 5.7})$$

$$x_2 = l_{cc} \cos (\alpha) - x_1 \quad (\text{Equation 5.8})$$

$$y_1 = x_2 \tan (\alpha) \quad (\text{Equation 5.9})$$

$$H_o = h - y_1 \quad (\text{Equation 5.10})$$

where x_1 and x_2 are intermediate parameters used in determining height y_1 and height H_o . As sintering progresses, x_2 changes over time and x_1 remains constant. Each unit cell can be represented by a regular hexagonal prism as shown in Figure 5.14. When viewed perpendicular to the XY-plane, the unit cell has a regular hexagonal surface area and is given by:

$$A_{hex} = \sqrt{3} \left(\frac{1}{\sqrt{3}} \times \frac{l_{cc}}{\sqrt{3}} \times \frac{l_{cc}}{\sqrt{3}} \tan 30^\circ \right) \quad (\text{Equation 5.11})$$

5.2.2.2 Volume of a Unit Cell

Considering the overlapping region between two neighboring layers shown in Figure 5.14, each layer of microspheres shares the overlapping region equally with the neighboring layer. Hence the total volume of one unit cell, V_{unit} is the sum of the full

Figure 5.76: Geometry of the shaded plane from figure 5.10b

Figure 5.77: Unit cell representation of a HCP microsphere assembled structure

volume within the non-overlapping region and half of the volume of the overlapping region from both the top and bottom unit cell respectively:

$$\begin{aligned}
 V_{unit} &= A_{hex} \times \left[2y_1 + \left(2 \times \frac{H_o}{2} \right) \right] \\
 &= A_{hex} \times (2y_1 + H_o) \\
 &= A_{hex} \times (h) \\
 &= \frac{3l_{cc}^3}{2} \tan(30^\circ) \sin(54.74^\circ) \\
 &\approx 0.707 l_{cc}^3
 \end{aligned} \tag{Equation 5.12}$$

Assuming no material loss during the sintering process, the volume of space within the unit cell that is occupied by a microsphere can be derived. This value corresponds to the scaffold porosity, p_s .

$$\begin{aligned}
 p_s &= \frac{\frac{4}{3} \pi r_o^3}{0.707 l_{cc}^3} \\
 &\approx 5.925 \left(\frac{r}{l_{cc}} \right)^3
 \end{aligned} \tag{Equation 5.13}$$

Equation 5.13 predicts the local porosity of the scaffold (which corresponds to the packing density of the microspheres) based on microsphere radius, r_o and center to center distance l_{cc} after sintering for a period of time, t . The important assumption used in the derivation of this formula is that the microspheres were perfectly ordered in a 3D HCP structure with no stacking faults and defects. Theoretically, the packing density at $t = 0$ (no necking) is 0.74. At this point, l_{cc} is equal to the diameter of a single microsphere ($2 r_o$), and $r = r_o$. These values, using Equation 5.13 would result in packing density of 0.7406.

5.2.3 Compensation for Local Porosity

The ideal case of a perfectly packed microsphere structure in hexagonal arrangement would have a packing density (corresponding to the scaffold porosity) of 0.74. In reality, stacking faults e.g., grain boundaries, misaligned regions etc. are common in

opal structures which result in a packing density below the theoretical ideal value of 0.74 (see Figure 5.15). These have to be taken into account in the modeling for local porosity, which is the percentage of void volume of the regions of the scaffold not inclusive of the channels.

5.2.3.1 Imperfect Stacking

Figure 5.16 illustrates a typical case of a stacking defect. The spaces between the non-contacting microspheres will be occupied by the scaffold material during the casting process. Thus, this space will result in a lower scaffold porosity than the ideal case of 0.74. During the sintering process, the structure experiences shrinkage due to densification. As such, this volume of space tends to decrease over time. The amount of shrinkage of this space can be assumed to scale to the amount of shrinkage. At $t = 0$ when there is no necking, the unit cell volume is at its maximum at $V_{unit\ max}$ and the center-to-center distance $l_{cc\ max}$, is simply two times the radius of a microsphere, $2r_o$. Thus, the final bulk volume (after sintering) of the overall structure containing a

Figure 5.78: SEM image of aligned 88 μm microspheres showing stacking faults (indicated by arrows)

Figure 5.79: Illustration of a simple stacking defect

total no. of m unit cells can be expressed as a percentage of the initial bulk volume at $t = 0$:

$$\frac{mV_{unit}}{mV_{unit \max}} = \frac{0.707l_{cc}^3}{0.707l_{cc \max}^3}$$

$$\frac{V_{unit}}{V_{unit \max}} = \left(\frac{l_{cc}}{l_{cc \max}} \right)^3 \quad (\text{Equation 5.14})$$

Equation 5.14 shows that after a period of sintering time, t , the shrinkage of the volume of the scaffold scales according to the cube of the ratio of the center-to-center distance at time t and $t = 0$. Thus, the amount of shrinkage of the space caused by stacking faults can be assumed to scale accordingly. This extra space can be found experimentally by subtracting the porosity derived from the scaffold at time t close to zero, P_{actual} from the ideal value of 0.74. For this research, this value $(0.74 - P_{actual})$ is termed ‘porosity compensation’ or P_{comp} and is to be subtracted from the theoretical value derived from Equation 5.13 at the respective time, t . As discussed previously, this value decreases over time due to shrinkage of the overall microsphere structure and its value at time t , can be expressed as:

$$p_{comp} \left(\frac{l_{cc}}{l_{cc \max}} \right)^3$$

Thus, taking this into account in Equation 5.13, the local porosity of the scaffold after considering the extra space caused by stacking faults can thus be expressed as:

$$p_s = 5.925 \left(\frac{r}{l_{cc}} \right)^3 - p_{comp} \left(\frac{l_{cc}}{l_{cc \max}} \right)^3 \quad (\text{Equation 5.15})$$

The variable of Equation 5.15 is l_{cc} which changes with time, t . The parameters r and $l_{cc \max}$ are fixed for a given microsphere size and p_{comp} can be determined experimentally.

5.2.3.2 Non-Contact Points of Individual Microspheres

The number of necks, or fusion between the microspheres must also be considered. The modeling of the unit cell volume in the previous section assumed a fully connected microsphere backbone: each microsphere touches and forms necks with 12 neighboring microspheres in a HCP structure. However, preliminary investigations on a sintered microsphere structure aligned via ultrasound agitation showed that the number of necks per microsphere is often less than 12 (see Figure 5.17). In this scenario, the total unit cell volume must be compensated from the volume decrease that would be caused by the necking region in the case of a microsphere with 12-contact points. This is illustrated in 2D in Figure 5.18. For an ideal case where a microsphere is able to establish 12 contact points, the unit cell volume can be found using Equation 5.12. However, in the case of a microsphere with one non-contact point, the extra volume must be added to Equation 5.12. This extra volume is represented by the orange region shown in Figure 5.18.

Figure 5.80: SEM image of HCP aligned 88 μm polystyrene microspheres (Arrows represent locations where there are no necking between microspheres)

Figure 5.81: 2D illustration for volume compensation of a non-contacting point of a microsphere

The total number of necks of a microsphere, N_{neck} formed during the sintering process at a specific time, t can be determined experimentally. The extra volume formed from non-contact points, can be determined by subtracting the unit cell volume at time, t from $V_{unit\ max}$, and adding back the fraction of the volume that should be occupied by the contacting points:

$$(12 - N_{neck}) \left(\frac{V_{unit\ max} - V_{unit}}{12} \right)$$

Thus, the actual volume of a unit cell, V_{actual} after considering the non-contact points of the microsphere can be expressed as:

$$V_{actual} = V_{unit} + (12 - N_{neck}) \left(\frac{V_{unit\ max} - V_{unit}}{12} \right) \quad (\text{Equation 5.16})$$

Therefore, the final local porosity of the scaffold, after taking into consideration the volume of space caused by stacking faults and the extra volume arising from non-contacting points of a microsphere, can be expressed as:

$$p_s = \frac{V_{microsphere}}{V_{actual}} - p_{comp} \left(\frac{l_{cc}}{l_{cc\ max}} \right)^3$$

$$p_s = \frac{22.63\pi_o^3}{12l_{cc}^3 + (12 - N_{neck})(l_{cc\ max}^3 - l_{cc}^3)} - p_{comp} \left(\frac{l_{cc}}{l_{cc\ max}} \right)^3 \quad (\text{Equation 5.17})$$

where $V_{microsphere}$ is the volume of a microsphere. The accuracy of this equation in determining the porosity of a scaffold fabricated via the inverse approach will be verified experimentally in the following chapters.

5.2.4 Global Porosity

Global porosity takes into account the porosity of the overall scaffold structure: that is inclusive of the channels (see Figure 5.7). The total volume of the channels (corresponding to the struts of the inverse mold) can be easily determined using

Pro/Engineer software. For strut sizes designed at less than 600 μm , it is important to convert to the actual strut size using the chart in Chapter 3 Figure 3.24 (e.g., a strut diameter designed at 400 μm would have an actual fabricated diameter of 600 μm) and use this value in the determination of the total channel volume. To calculate the overall scaffold porosity, it is first necessary to obtain the fraction of the total volume

of the scaffold that is occupied by the channel network, $\frac{V_c}{V_s}$, where V_s is the bulk volume of the scaffold and V_c is the total volume of the channels (or struts in the mold). The global porosity, P_g can be expressed as:

$$\begin{aligned} P_g &= \frac{p_s(V_s - V_c) + V_c}{V_s} \\ &= p_s - \frac{V_c}{V_s}(p_s - 1) \end{aligned} \quad (\text{Equation 5.18})$$

In Equation 5.18, the term $(p_s - 1)$ will always give a negative value. Thus, the greater the volume of the channel network, V_c , the larger is the global porosity, P_g of the overall scaffold.

5.3 Chapter Summary

Theoretical modeling of the scaffold structure has been described in this chapter. The modeling was based on structural characteristics of the inverse mold: the neck length, angle of intersection of the necking plane, and packing density of the overall microsphere structure. These correspond to various aspects of the final scaffold architecture. Frenkel's sintering model, which applies to the early stages of sintering, will be used to predict the neck radius of the microspheres (corresponding to the size of interconnection between scaffold pores). Based on the assumption of a perfectly ordered HCP microsphere structure, an Equation that correlates local porosity with center to center distance l_{cc} , which is in turn dependent on sintering time, was derived (Equation 5.13). Porosity compensation was also considered for an imperfect microsphere structure with stacking defects (Equation 5.17). Finally, an equation for

global porosity of the overall scaffold which takes into account the porosity created by the channel network was presented (Equation 5.18). The next chapter will outline the experimental procedures in fabricating the scaffold using the inverse mold as well as the methods used to investigate the validity of the theoretical models derived in this chapter.

Chapter 6 – EXPERIMENTAL PROCEDURES

This chapter discusses the procedures of the experiments carried out in this research. These include the fabrication of the scaffold using the inverse mold and the characterization studies on the physical structure of the fabricated scaffold (in terms of pore size, pore interconnectivity, local porosity and global porosity). Preliminary *in vitro* studies on the scaffolds are also explained, along with the methods of cell culture and maintenance protocols and assessment procedures on the cytotoxicity of the inversely fabricated scaffolds.

6.1 Scaffold Fabrication

Scaffolds are produced using the proposed inverse fabrication method with gelatin type B as the scaffold material. Unlike synthetic materials, gelatin is a naturally derived protein polymer and is a structural protein present in the ECM. It tends to have a more native surface relative to synthetic polymers in terms of biological interaction with cells. Being a widely investigated ECM component, it has tremendous potential in both soft and hard tissue engineering. In principle, the inverse fabrication method can also be employed using other scaffold materials including PLA, PLGA and poly-urethanes (PU) as long as the solvent and scaffold material compatibility requirement is fulfilled (as discussed in chapter 4). The concept behind the inverse fabrication method is based on the lost mold technique whereby the scaffold is obtained upon the removal of the mold. Previous lost-mold based fabrication techniques have focused only on using the mold to create the macro-channels of the scaffold structure. In this research however, the architecture of the inverse mold represents the negative replica of the scaffold structure and thus shapes the entire pore architecture of the scaffold including pore size, pore interconnectivity, geometry, shape, porosity, channel size and configuration. The inverse mold is comprised of polystyrene microspheres and an RP material. Thus, the entire fabrication procedure (from the mold to the scaffold) will differ from previous lost-mold methods that require only an RP material. For this scaffold fabrication concept to become a reality, a proper fabrication protocol is necessary. The process flow for

the inverse fabrication method is shown in Figure 6.1 and will be outlined in the following sections.

Figure 6.82: The various processes involved in the inverse scaffold fabrication method

6.1.1 Mold Preparation

One of the key components in the inverse scaffold fabrication process is the inverse mold, of which the related fabrication methodologies have been studied in Chapter 4. This mold comprises of an RP fabricated mold filled with mono-dispersed polystyrene microspheres. The RP component is used to create the channels of the

scaffold while the microspheres control the micro-scale features such as pore size, pore interconnectivity, etc. The RP mold is eventually filled with microspheres and fused together via sintering to form the inverse mold which corresponds to the ‘negative’ of the entire scaffold architecture. In order to create the scaffold channels, the inverse mold must consist of interconnected struts so that upon mold removal, the spaces left behind by the struts form the channels within the scaffold. However, the solvent used to dissolve the RP mold is able to dissolve the gelatin material as well. Thus, the RP mold cannot be used directly as the inverse mold. A two-step lost mold approach is employed: after the RP mold is fabricated, a second mold material is infiltrated into the RP mold. The RP mold is subsequently removed to obtain the second mold (see Figure 6.2). The material for the second mold must be such that it can be removed in solvents that do not dissolve gelatin.

Figure 6.83: Illustration of fabricating the second mold

6.1.1.1 RP Mold Design

The architecture of the RP mold was designed using a commercial CAD (Computer-Aided-Design) software, Pro-Engineer (Pro-E). Since the RP mold cannot be used directly as the inverse mold and a two-step lost mold method has to be used, the RP

mold was designed to consist of interconnected channels instead of struts. In this manner, the struts which are necessary to form the scaffold channels will be created in the second mold; that is after the second mold material has infiltrated the RP mold and then subsequently removed.

As a simple demonstration on using RP molds to create scaffold channels, a mold design with a basic channel configuration is adopted. The mold design consists of a square compartment enclosing a 3D network of interconnected channels aligned in the X-, Y- and Z- axis as shown in Figure 6.3. From the figure, the square compartment is located at the center portion of the mold and is designed in such a way to prevent the second mold material from flowing out of the RP mold during the infiltration process. From studies done in Chapter 4, the height of the square compartment is designed to be less than the outer rim as illustrated in Figure 6.2 such that the resulting LDPE mold has a base thickness approximately similar to that of a glass slide. This is to maximize the stacking effect of the microspheres within the LDPE mold. Based on studies conducted in Chapter 3, the diameter input for the Pro-E software is set at 600 μm so that the actual fabricated value does not differ too much from the designed value.

Figure 6.84: CAD drawing of RP mold with 3D, top and side view

6.1.1.2 RP Mold Fabrication

The CAD data of the RP mold is exported as a STL (stereolithography) format file which is the *de facto* standard in all RP systems. The file was then processed using the Objet application software, Objetstudio™, which allows the user to place and orientate the parts to be printed (see Figure 6.4) as well as to define the building parameters such as layer thickness and printing speed. The mold was positioned in a way such that the x- and y- axis channels are aligned diagonal to the print axis. This is to minimize the deviation in channel diameter due to the printing methodology of the Objet printer (as explained in Chapter 3).

The RP mold was fabricated using the Objet printer (Eden350v) with their proprietary UV curable photopolymer, FullCure® 720 as the model material and FullCure® 705 as the support material. Upon completion of the printing process, the RP mold is stored in 1M NaOH for 24 hours to dissolve away the support material. The mold is subsequently rinsed in DI water to remove the NaOH.

Figure 6.85: Objet studio software

6.1.1.3 Second Mold Fabrication

In this research, low density Polyethylene (LDPE) was used as the second mold material due to the following reasons:

- It has a melting temperature higher than the glass transition temperature polystyrene so that it does not melt during microsphere sintering.
- The solvent used to dissolve LDPE does not affect the gelatin material.
- The solvent used to dissolve the RP mold does not dissolve the LDPE mold.

LDPE pellets were placed onto the RP mold and melted at 130 °C in a vacuum oven for 24 hours. At this temperature, the LDPE material melts and begins to fill up the spaces of the RP mold including the channels. As the melted LDPE is viscous in nature, the infiltration was assisted under vacuum environment. Additional LDPE pellets were periodically added on top of the LDPE melt until the melt reached the brim (see Figure 6.5). Finally, the RP mold was removed by immersing the LDPE-infiltrated mold into a beaker containing a glycol-based solvent, Dynasolve 2000 (as per recommendation from the Objet supplier) to obtain the second, LDPE mold with interconnected struts. This process was carried out in an oven at 60 °C. The LDPE

mold is then rinsed with sufficient water to remove the glycol-based solvent and stored for future use.

Figure 6.86: Fabrication of the second mold: A) RP mold placed in oven, B) RP mold filled with LDPE pallets, C) LDPE pallets melted at 130 °C, D) Melted LDPE casted into the RP mold

6.1.1.4 Inverse Mold Fabrication

The final, inverse mold comprises of the LDPE mold filled with monodispersed polystyrene microspheres. Based on studies conducted in Chapter 4, ultrasound agitation will be used to assemble the microspheres into an ordered, 3D arrangement within the mold. This was conducted in an ultrasonic bath as shown in the setup in Figure 6.6. The LDPE mold was placed on top of a holder whereby the water level of the ultrasound bath is just touching the bottom of the mold. A 50 μl suspension of 88 μm diameter polystyrene microspheres in isopropanol (IPA) solution was dispensed drop-wise into the LDPE mold at 1 minute intervals while being continuously subjected to agitation. Note that it is difficult to gauge the exact amount of IPA needed to fill the mold as the amount of microspheres being drawn into the pipette tip using the pipettor is random. The mold is assumed to be completely filled when microspheres can be seen moving out from the rim of the mold where it forms patches

of HCP aligned microspheres (see Chapter 4 Figure 4.17). After this final addition, the setup was left to stand for 12 hours, allowing the IPA to evaporate at ambient conditions. Finally, the mold is transferred to an oven and sintered at 110 °C to fuse the entire structure together.

As discussed in Chapter 5, the sintering interval will have a profound effect on the pore structure of the scaffold. To investigate the effect of sintering time on scaffold global porosity and interconnection size between pores, molds were prepared and sintered at the following time intervals which were chosen arbitrarily: 2, 8, 16, 24, 32, 40, and 48 hours.

Figure 6.87: Ultrasound bath setup for microsphere assembly

6.1.1.5 Mold Preparation for Local Porosity Test

For local porosity tests, it is not necessary for scaffolds to have a channel network within its pore structure. Hence, scaffolds will be prepared without channels and measured for its porosity. The fabrication concept in this section is similar to the inverse method of producing scaffolds with channels. However, instead of creating an inverse mold using microspheres and a RP mold, only microspheres will be used. The setup is shown in Figure 6.7. A glass slide affixed to a polypropylene ring that was manually cut from a cryotube was used in place of the LDPE mold. Similar to the procedure in Section 6.1.1.4, a 50 μ l suspension of polystyrene microspheres in

isopropanol (IPA) solution was dispensed drop-wise from the top of the polypropylene ring at 1 minute intervals while being subjected to ultrasound agitation. After evaporation of the IPA, the mold was transferred to the oven and sintered at 110 °C at the time lengths mentioned in section 6.1.1.4. The sintered microsphere template was then carefully removed from the polypropylene ring and stored for future use. To compare the differences in local porosity, microsphere templates were prepared in a similar manner but without using ultrasound agitation to induce microsphere alignment. Due to the limited amount of 88 μm polystyrene microspheres, these templates were sintered at 3 time intervals: 8, 16 and 24 hours.

Figure 6.88: Ultrasound setup for microsphere template fabrication

6.1.2 Mold Casting

The next step was to fill up the inverse mold with the scaffold material. Following a published method, a 3 % gelatin (type B, Porcine skin, bloom 225, Sigma Aldrich) solution was prepared by dissolving 0.3 grams of gelatin type B powder in 10 ml of double distilled water at 37°C. As the inverse mold was densely packed with microspheres, the infiltration of the scaffold material into the mold can be a difficult process. The gelatin will not infiltrate the mold by simply dispensing the gelatin solution in a drop-wise manner onto the mold (see Figure 6.8a). Instead, it forms a gelatin droplet that remains at the top of the mold. It is essential that the gelatin solution infiltrates the mold entirely and fills up the internal spaces so as to accurately

replicate the internal mold architecture. Ultimately, this corresponds to the resulting scaffold pore structure.

To allow infiltration of the scaffold material, the gelatin solution was adjusted to contain 40% ethanol. This allows the solution to easily seep into the mold, filling up the spaces within (see figure 6.8b). However, for molds that were sintered for more than 32 hours, it was observed that the gelatin solution with 40% ethanol was still unable to infiltrate into the mold. A higher ethanol concentration will be required. This is due to the densification process of the microspheres during sintering. The longer the sintering time, the smaller the spaces between the microspheres and hence, the harder it is for the gelatin solution to seep into the mold structure. The recommended ethanol concentrations that will allow the gelatin to infiltrate the mold at specific sintering time lengths were recorded through experiments and tabulated in Table 6.1:

Table 6.4: Recommended ethanol concentrations

Sintering time (hrs)	1	8	16	24	32	40	48
Ethanol concentration(%)	20	20	40	40	50	50	65

Immediately after the casting process, the gelatin-infiltrated molds were frozen at -80°C overnight.

Figure 6.89: Scaffold material casting using gelatin type B solution with A) 0% ethanol and, B) 40% ethanol

6.1.3 Mold Removal

The inverse mold has to be removed and the resulting scaffold structure dried to obtain the scaffolds for experimental studies. At this point, it was necessary to consider the approach on both removing the mold and drying the scaffold without damaging its pore structure. Drying methods e.g., freeze drying, oven drying etc are known to result in shrinkage and/or collapsed structures due to surface tension forces associated with the drying process . Therefore, a crosslinking process was investigated. It was incorporated in the fabrication procedure to strengthen the mechanical properties of the scaffold structure. The procedures involving these processes are outlined next.

The frozen molds were first placed in a freeze dryer and lyophilized at -56°C for 24 hours to remove the water content. Next, the dried molds can either be transferred to a vacuum oven for dehydrothermal crosslinking or processed by solvents to remove the mold. For dehydrothermal crosslinking, the mold (dried) was transferred to a vacuum oven and heated at 100°C and 30mTorr for 24 hrs. Both LDPE and polystyrene material can be dissolved in heated toluene. The mold construct was immersed in toluene at 75°C for 48 hrs with 6 changes of toluene in between to allow the inverse mold to completely leach out of the scaffold structure. At this temperature, the LDPE mold as well as the polystyrene microspheres were dissolved. To observe the effects of dehydrothermal crosslinking on the scaffold structure, mold removal was also carried out immediately without crosslinking. The 3 different process routes undertaken in this section are listed in Table 6.2.

Table 6.5: Process routes for mold removal

Process sequence		
Method 1	Method 2	Method 3
1) Freeze drying of frozen mold	1) Freeze drying of frozen mold	1) Freeze drying of frozen mold
2) Crosslinking	2) Mold removal	2) Mold removal
3) Mold removal	3) Scaffold drying	3) Scaffold drying
4) Scaffold drying	4) Crosslinking	
	5) Scaffold drying	

6.1.4 Scaffold Drying

Once the mold removal process has been completed, the scaffolds were immersed in 100% ethanol for 24 hours with 6 changes in between to remove residual toluene. Three methods were explored to dry the scaffolds: oven drying, freeze drying and critical point drying. For scaffolds to be dried via oven drying, the scaffolds were placed onto a filter paper and transferred to an oven and heated at 70°C for 12 hours. For freeze drying, the scaffolds were first frozen at -80°C and transferred to the freeze dryer. The drying process was carried out at -56°C for 24 hrs. Scaffolds to be dried via critical point drying were processed immediately after the mold removal step using 100% ethanol.

6.2 Scaffold Characterization

The physical structure of the gelatin scaffolds will be studied in this section. Scaffolds will be examined using scanning electron microscopy (SEM). The porosity of the scaffolds will also be measured using a simple experimental setup which will be outlined in the following sections.

6.2.1 Morphology of Scaffold Structure

The porous morphologies of the gelatin scaffolds were studied with scanning electron microscopy (SEM; S-3200N, Hitachi, Japan) at 15 kV. Scaffolds were carefully cut with a razor blade and coated with gold for 120 seconds using a sputter coater (Desk-II, Denton Vacuum Inc). The gas pressure was less than 50 mtorr, and the current was set to 20 mA. Each scaffold was examined for its pore size, interconnection size between pores, channels as well as the pore distribution.

6.2.2 Porosity Measurement

The porosity values of the gelatin scaffolds prepared from the inverse fabrication method were measured by a liquid displacement technique, similar to the published method by Zhang and Ma , Hsu *et al* and Guan *et al* . Scaffold samples were individually immersed in a cylinder containing a known volume of ethanol (V_1). The sample was immersed for 1 minute to allow the ethanol to seep into the scaffold pores. At this point, the total volume of ethanol and the ethanol-infiltrated scaffold was recorded (V_2). The scaffold was then removed from the cylinder and the residual ethanol volume was recorded (V_3). The volume difference ($V_2 - V_1$) represents the volume of the gelatin scaffold while ($V_1 - V_3$) represents the volume of the ethanol being held in the pores by the gelatin scaffold. Thus, the bulk volume of the scaffold (including the pores) can be expressed as:

$$\begin{aligned} V &= (V_2 - V_1) + (V_1 - V_3) \\ &= V_2 - V_3 \end{aligned} \quad (\text{equation 6.1})$$

The experimental porosity, P_e of the scaffold can then be derived:

$$P_e = \frac{V_1 - V_3}{V_2 - V_3} \quad (\text{equation 6.2})$$

6.2.3 Measurement of Number of Necks on Microspheres

Microsphere templates were created following the methods in Section 6.1.1.5 using 160 micron sized polystyrene microspheres. The templates were sintered for different time lengths: 1hr, 8hrs, 16hrs, 24hr, 32hrs, 40 hrs, and 48 hrs. After the sintering process, the necks between the microspheres were broken up by gently pressing the template. The microspheres obtained were next attached to a glass slide that was affixed with a duct tape and examined using SEM. It is important to constantly tilt and rotate the microscope stage that was holding the glass slide in order to see the necks on the entire surface of the microsphere. The necks were characterized by rough flat surfaces shown in Figure 6.9. The microspheres that were examined are those that had completely detached off the template and were not attached to any neighboring microspheres. A total of 50 microspheres were observed from each template and the number of necks in each microspheres was then counted for all sintering time intervals and tabulated.

Figure 6.90: Polystyrene microsphere showing the neck regions (see arrows)

6.3 Preliminary *In Vitro* Characterization

This section covers the investigative work for *in vitro* cell responses to fabricated scaffolds. The scaffolds were assessed for cytotoxicity and also their propensity to encourage cell proliferation. Before going into the details of these experiments, the methods and protocols concerning cell culture work will first be discussed

6.3.1 Cell Culture Process

The main objective of culturing cells is to obtain viable cells in sufficient quantities for experiments. Several processes are involved in growing/maintaining the cells: Cell thawing, passaging, freezing/banking etc. The process flow of cell culture are illustrated in Figure 6.10.

Figure 6.91: Process flow of cell culture

6.3.1.1 Cell Culture Reagents and Equipments

Cell culture work were carried out in a Gelman Class II Biosafety cabinet with vertical laminar flow, to allow protection for the cell culturist and the cells. A pair of rubber gloves and lab coat were worn at all times. Cells were cultured in tissue culture flasks (25 cm² and 75 cm² size available) which were stored in an incubator that was maintained at 37°C and 5.0% CO₂ atmosphere.

Basic reagents required for cell culture are listed below :

1. Dulbecco's Modified Eagle Medium (DMEM) (invitrogen)
This is the supplement medium for cells which contains essential inorganic salts, amino acids, vitamins and buffers which are important for cell metabolism and division. Additionally, it consists of 10% Foetal Bovine Serum (FBS) to provide the growth factors required for cell growth and 1% antimycotic solution, an antibiotic that prevents bacterial growth and microorganism contamination.
2. 0.25 wt% trypsin-ethylene diamine tetraacetic acid (EDTA)
When cells come into contact with the surface of the culture flask, they form bonds and attached to the surface. Cells tend to attach to a surface so they may

spread and undergo cell division. Once they have reached confluency, they must be passaged into other flasks for them to multiply. Trypsin is used to detach the cells from the surfaces of the flask so that they may be collected and passaged into other flasks.

3. Phosphate Buffered Saline (PBS)

PBS is an isotonic buffer solution with pH 7.44 containing a number of mineral salts that are non-toxic to cells. They are usually used for washing and cleaning the cells from metabolic waste and cell debris that may be present in culture flasks.

6.3.1.2 Cell Thawing

Thawing is the process whereby frozen cells are resuscitated for usage after a period of storage in liquid nitrogen environment. Upon collection of the vial of frozen cells from the supplier, the cells were resuscitated by rapidly thawing them in a water bath at 37°C. The vial was carefully immersed in the water bath with the water level just reaching the cap. This is to prevent the water from seeping into the vial which may contaminate the cells. After thawing, the vial of fibroblasts was handled as follows:

1. The contents of the vial was emptied into a 15 ml centrifuge tube containing 5ml of DMEM using a micro-pipette.
2. The tube was then centrifuged at 2500 rpm for 3 minutes. During this process, the cells were collected at the bottom of the tube and formed a cell pellet.
3. The supernatant contains the storage medium that was in the vial and was carefully removed using a micro-pipette without disturbing the cell pellet.
4. Fresh DMEM (5ml) was dispensed into the tube and re-suspended using a micro-pipette to break up the cell pellet.
5. The contents were transferred into a 25 cm³ culture flask using a pipette gun.

The flask was transferred and stored in an incubator. Upon reaching confluency, the cells may be passaged into more flasks, of which details will be discussed next.

6.3.1.3 Cell Passaging

Cell passaging or splitting is the process of multiplying the cells into sufficient quantities required for experiments. Confluency refers to the state at which the cells have formed a continuous monolayer and covered the surface of the flask (see Figure 6.11b). Upon reaching near-confluency, the cells will be collected and transferred into new flasks so there will be sufficient space for them to divide and grow. The sub-cultivation ratio was 1:3, meaning that the cells collected in one flask will be equally divided into three flasks. The passaging procedures are as follows:

1. The old DMEM was removed from the flask. The flask was added with 5 ml of PBS and slightly agitated for a few seconds to wash the cells. The PBS was subsequently removed from the flask.
2. 4 ml of trypsin was dispensed into the flask and placed into the incubator for 5 minutes in order to detach the cells. The flask was microscopically examined to see if the cells have detached. This can be verified by gently shaking the flask and observing if the cells are flowing on the surface. If not, the flask will be transferred back to the incubator for one more minute until the cells have completely detached.
3. Once the cells have detached, 10 ml of DMEM was dispensed into the flask to neutralize the trypsin. The contents in the flask were transferred to a 15 ml centrifuge tube and centrifuged at 2500 rpm for 4 minutes.
4. The supernatant was carefully removed without disturbing the cell pellet.

Figure 6.92: Layer of fibroblast cells A) near-confluent layer of cells with spaces between individual cells, B) confluent monolayer of cells

5. 1 ml of DMEM was dispensed into the tube and subsequently re-suspended to break up the cell pellet.
6. The 1 ml cell suspension was equally divided into 3 flasks using a micro-pipettor. Each flask was added with fresh DMEM until the required amount is reached (15 ml for 75 cm³ flask and 5 ml for 25 cm³ flask).

The flasks were transferred and stored in the incubator to allow the cells to divide and grow. This process of passaging and growing the cells will ensure a continuous supply of cells for experimental studies.

6.3.1.4 Cell Freezing

Cell freezing/banking is whereby additional cells are stored for future usage. The procedures are almost similar to passaging: cells are washed, then detached from the flask using trypsin and centrifuged to obtain a cell pellet. The supernatant was removed from the centrifuge tube and replaced with a cryoprotectant medium which is made up of DMEM and 8% (v/v) dimethyl sulfoxide (DMSO). The contents were resuspended to break up the cell pellet and transferred to a cryotube. The cryotube was stored in a -80°C freezer before being transferred to a liquid nitrogen storage tank.

6.3.1.5 Cell Counting

The main objective of cell counting is to obtain the cell density for a known volume of cell suspension. As each individual flask contains millions of cells, a method is necessary to count them so that the desired amount can be drawn correctly each time for experiments based on the calculated density. Cell counting was performed using the haemocytometer (Marienfield, Germany), a modified glass slide with nine square grids (each 1 x 1mm with 0.1 mm depth) engraved on it (see Figure 6.12). The haemocytometer was sterilized with 70% ethanol before usage. After the cells were detached from the surface, centrifuged and resuspended, 10 µl the cell suspension was transferred to a cryotube and mixed with 10 µl of Trypan blue solution (Sigma, Catalog no. T8154) and PBS to dilute the cell suspension. Subsequently, 10 µl of the

diluted cell suspension was then transferred to the haemocytometer. Cells that were alive appeared clear and unstained while dead cells would absorb the trypan blue dye and stained blue. The cells located within the A, B, C, D, E square grids were counted. From this number, the cell density can be obtained as follows:

1. Dilution factor, $d = (\text{amount of PBS} + 20)/10$

2. Cell density = $\frac{\sum N}{5} \times d \times 10^4 \text{ cells / ml}$

where $\sum N$ is the total number of cells counted from the 5 square grids. Note that it is not possible to obtain the exact cell density and this calculated cell density is only a close approximation

Figure 6.93: a) Haemocytometer, B) Haemocytometer counting grids

6.3.1.6 DNA Cell Quantification

Cell numbers are measured using a DNA quantification assay kit (Picogreen, Molecular Probes, invitrogen) . It is a highly sensitive assay whereby its dye is specific only to double stranded DNA and emits a green fluorescence upon its binding to DNA. Hence, the intensity of the fluorescence emitted can be directly correlated to the number of cells.

A reference cell standard calibration curve was tabulated for converting the sample fluorescence intensity to cell numbers. Similar to previous methods, cells were detached, centrifuged and counted. Upon determining the cell density of the cell suspension, 200,000 cells/ml was created and stored in a centrifuge tube. This tube

was centrifuged again to remove the supernatant. After subjecting the cell pellet to a freeze-thaw cycle at -80°C and 37°C respectively, 1.0 ml of lysis buffer was added and the lysate was resuspended by brief vortexing and used as the stock solution. The freeze-thaw cycle assisted in permeabilizing the membrane of the cells while the subsequent addition of lysis buffer ensured the full access of cellular nucleic acids to the Picogreen dye. To quantify the cell number, a cell dilution series ranging from 20 to 20,000 cells (see Table 6.3) were created from the 200,000 cells/ml centrifuge tube. Each dilution was stored in separate cryotubes. For example, 100 μl drawn from the centrifuge tube and adding 900 μl of DI water will result in 20,000 cells in 1 ml of stock solution. Drawing another 10 μl from the tube and adding 990 μl of DI water will result in 2,000 cells in 1 ml of stock solution and so on. 100 μl of each dilution was transferred into a 96-well microplate (black flat-bottom, Greiner, Germany) and each well was added with 100 μl of Picogreen working solution (dilution 1:100) to final volumes of 200 μl per well. Blanks of 200 μl wells without cells (100 μl of DI water and 100 μl of Picogreen working solution) were also prepared for fluorescence measurement. An additional dilution series ranging from 40 to 40,000 cells were prepared using the same protocol.

Table 6.6: Cell dilution series

Volume of DI water (μl)	Volume of stock solution (200,000 cells/ml of lysis buffer)	Final cell density (cells/ml)	Final cell number per well (100 μl from each dilution)
1000	0	0	0 (blank)
999	1	200	20
990	10	2,000	200
900	100	20,000	2,000
0	1000	200,000	20,000

The 96-well microplate was further incubated for 5 minutes after which the fluorescence intensity of each well was quantified using a CytoFlour 2350 fluorescence microplate reader (PerSeptive Biosystems) at excitation and emission wavelengths of 485 and 535 nm respectively. Finally, a standard cell calibration curve was generated by plotting the measured fluorescence values versus cell number.

6.3.2 Cytotoxicity Assessment of Scaffolds

A good tissue engineering scaffold requires that it does not elicit any adverse cellular responses when seeded with cells or when implanted into a host. However, the gelatin scaffolds fabricated in this research using the inverse fabrication method are involved in a number of processing steps and these may induce toxicity within the scaffolds during the course of fabrication. It is important to ensure that the fabrication process does not cause the scaffolds to become contaminated with toxic substances, and thus the need for cytotoxicity assessment. The cell type used for the cytotoxicity assessment was fibroblast (NIH/3T3, ATCC: CRL-1658) purchased from American Type Culture Collection (ATCC). Fibroblasts are commonly used cells for standard cytotoxicity testing of materials and are well documented in Practice F 813, Test Method F 895, and ISO 10993-5.

6.3.2.1 Extract Dilution Cytotoxicity Test

To assess the biological response of cells in reaction to possible toxic leachables derived from the scaffolds, a cytotoxicity test was performed using the extract dilution method. This method involves the exposure of cell monolayers which are grown to near confluence in flasks to fluid extracts obtained from scaffolds. The cells were subsequently evaluated for signs of toxicity such as changes in the size or appearance of cellular components or a change in cell viability.

6.3.2.2 Extract Preparation

The fluid extracts were prepared as outlined in ISO 10993-5. Briefly, the gelatin scaffolds (one made from the inverse mold, and one from the microsphere template) was immersed in separate tubes of 15 ml of DMEM and incubated at 37°C for 72 hrs. Using the same procedure, extracts were obtained from a small piece of latex rubber (Velos Perforex, Manchester, UK) as a positive control. Latex is known to have a strong cytotoxic effect leading to extensive cell death and lysis, and is therefore commonly used as a positive control for cytotoxicity tests.

6.3.2.3 Cytotoxicity Test Procedure

3T3 fibroblasts were seeded onto 24-well plates (transparent, flat-bottom, Greiner, Germany) at a concentration of 5×10^3 cells per well and incubated at 37°C for 24 hours. Subsequently, the culture mediums in all cell-seeded wells were separately replaced using fluid extracts derived from the gelatin scaffolds and positive control (n = 5 wells each). As a negative control, fresh DMEM was used in the test (n = 5). The well plates were returned to the incubator to allow the cells to respond to the extracts and controls. Picogreen assay (Molecular Probes, Invitrogen) was used to evaluate the cell number in each well after being treated with the fluid extracts. This was conducted at day 1 (24 hours) and day 3 (72 hours) after adding in the fluid extracts. Briefly, all wells were cleared of culture mediums and subjected to a freeze-thaw cycle before addition of the lysis buffer. 100 μ l of the lysate was dispensed into a 96 well plate and added with Picogreen working solution (1:100 dilution) with a final working volume of 200 μ l per well. The fluorescence intensity of each well was measured and the corresponding cell number was obtained from the reference cell standard calibration curve and compared. Finally, the cells were also microscopically examined using trypan blue stain. Cells that are viable will not permit the dye to enter through their membrane. Otherwise, a dead cell will be stained blue in color.

6.3.3 Cell Proliferation Assay

One of the main functions of a tissue scaffold is to provide a temporary, 3D space for tissue development. In this aspect, seeded cells are required to proliferate until the scaffold/cell construct achieves a large enough cell mass suitable for implantation. The scaffold must therefore provide a conducive environment that encourages the proliferation of seeded cells. This section of the in vitro characterization work explores the propensity of the fabricated gelatin scaffolds in promoting cell proliferation. Details are outlined next.

6.3.3.1 Scaffold Samples

The proliferation test involves gelatin scaffolds with different pore architectures that were produced using the inverse fabrication method and these are described as follows:

- GS2: These are disc shaped gelatin scaffolds fabricated from microsphere templates. The templates were produced using 88 μm polystyrene microspheres and sintered for 2 hours. Overall scaffold size is 10 mm in diameter and approximately 3mm in height.
- GS16: Similar to GS1 but prepared from microsphere templates that were sintered for 16 hours.
- GS32: Similar to GS1 but prepared from microsphere templates that were sintered for 32 hours.
- GSC: These are square shaped gelatin scaffolds fabricated from the inverse mold. Thus it consists of a 3D interconnected network of 500 μm sized channels as well as spherical pores of uniform size. The sintering time used for the preparation of the inverse mold was 16 hours.
- CS: These are disc shaped gelatin scaffolds that were prepared using the conventional freeze drying method and were used as controls.

Fabrication of CS (control gelatin scaffolds) is straightforward. Briefly, a 3% gelatin solution was cast into an acrylic mold. The mold was then frozen at -80°C for 8 hours and dried using a freeze dryer at -55°C . Dimensions of the scaffold were measured as 15 mm diameter and 4 mm in height.

6.3.3.2 Scaffold Sterilization

Prior to cell seeding, the gelatin scaffold samples were sterilized with 100% ethanol at room temperature for 24 hours. The ethanol was removed by immersing in sterilized phosphate-buffered saline (PBS) on an orbital shaker (100 rpm) for 8 hours. The scaffolds were then soaked in DMEM for 24 hours to allow the serum proteins to adsorb into the scaffolds.

6.3.3.3 Cell Seeding

Cells were detached and centrifuged to obtain a cell pellet. After determining the cell density, the gelatin scaffolds (n=5 each) were placed in an empty 24-well plate. Care was taken to ensure that the scaffolds do not absorb too much culture medium before being placed onto the well as it may flow out of the scaffold upon contact with the surface of the well. This may induce the seeded cells to flow out of the scaffold and into the wells during the seeding process. The cells were seeded directly on top of the scaffold using a micropipettor with a small seeding volume of 40 μ l. 1,000,000 cells were seeded per scaffold. The cells were allowed to attach for 8 hours in the incubator at 37 °C and 5% CO₂-humified atmosphere. Thereafter, 1 ml of culture medium was carefully added into each well through the side of the well without disrupting the cell-seeded scaffold and the scaffolds were maintained in the incubator for a total culture period of 14 days. The culture medium was changed every 3 days. Samples were taken out after 1, 5, 10 and 14 days of cultivation for proliferation analysis.

6.3.3.4 Cell Proliferation Analysis

The proliferation of cells in the scaffolds was assessed using the Picogreen assay (Molecular Probes, invitrogen) following the procedure outlined in Section 6.3.1.6 with some slight modifications. The modifications are due to the fact that cells that are located in the scaffold interior will not be able to detach from the scaffold. Thus, the DNA located within the scaffold cannot be collected for quantification. To obtain the DNA inside the scaffold, a DNA extraction kit (DNeasy quantit) was used to dissolve the scaffold into its constituent protein units. Briefly, the culture mediums in all wells were removed and the scaffolds were transferred to a new well. This step is to allow the Picogreen assay to quantify only the DNA of the cells in the scaffold and not those that have attached to the well surface. Subsequently, all cell-seeded scaffolds were separately digested in a 1 ml solution of 0.0125% (w/v) papain containing 0.1M L-cysteine and 0.05M EDTA for 12 hours at 50 °C. This step was necessary as the scaffold must be fully digested in order to free the DNA from the cells located at the scaffold interior. To measure the fluorescence intensity, 100 μ l of the digested solution was transferred into a 96-well microplate (Corning) and added with Picogreen working solution to a final volume of 200 μ l per well. The

fluorescence intensity of each well was quantified using a CytoFlour 2350 fluorescence microplate reader (PerSeptive Biosystems) at excitation and emission wavelengths of 485 and 535 nm respectively. Finally, the readings were compared with the reference cell standard calibration curve to obtain the total number of cells located in each scaffold.

6.3.3.5 Statistical Analysis

Results obtained for cytotoxicity test and cell proliferation tests were expressed as means $\pm$ standard deviations. Assuming null hypothesis, the statistical significance between two sets of data was analyzed using two-population student's t-test. For cytotoxicity test, the statistical test was used to determine any significant difference in cell number for all scaffolds with respect to negative control. For cell proliferation tests, it was used to determine any significance difference in cell number between two scaffolds within the same measurement day. Data were taken to be significant when a P-value of 0.05 or less was obtained.

Chapter 7 – RESULTS AND DISCUSSIONS

This chapter discusses the details concerning the inverse scaffold fabrication procedure as well as the characterization results of the resulting scaffold pore structure. Furthermore, the results of the *in vitro* cytotoxicity assessment and proliferation assay are presented.

7.1 Inverse Scaffold Fabrication Procedure

This section documents the proper steps and procedures for the proposed inverse scaffold fabrication method based on experimental results.

7.1.1 Production of Inverse Molds

One of the important aspects of the proposed inverse fabrication method requires the use of a mold (the inverse mold) to form the entire architecture of the scaffold. As such, the structure of this inverse mold must be the exact negative replica of the entire scaffold structure. Two types of inverse mold were used in this research: 1) inverse molds without struts and, 2) inverse molds consisting of interconnected struts. The first type of inverse mold is made purely from microspheres which are assembled and fused together into a single template. They are used to create scaffolds that require no additional features within its structure. On the other hand, the second type of inverse mold is used to create scaffolds requiring specific architectures, e.g., a 3D, interconnected network of channels integrated within its porous structure. Similarly, the production of the second type of inverse mold uses microspheres but is not as straightforward and requires a two-step lost mold process involving RP fabrication (see Section 6.1.1.1 and Figure 6.2).

The RP mold and its CAD model as well as the second LDPE mold are shown in figure 7.1. The reason for this two step procedure is due to the difficulty in removing the RP mold without dissolving the scaffold material (gelatin). From SEM observation (see Figure 7.2 A), the strut size of the LDPE mold which was designed at 600 μm is close to the diameter of the actual fabricated strut size at 585 μm ,

suggesting that the LDPE material has completely fill up the spaces of the RP mold. However, the actual scaffold channel diameter appears to smaller than the strut size of the LDPE mold (see Figure 7.2B). The second type of inverse mold was created by filling up the LDPE mold with mono-dispersed polystyrene microspheres using the ultrasound agitation method and then sintered at 110°C to fuse the whole structure in place (see Figure 7.3). From observation, the sintered inverse mold appears to be stable and fixed in place as there are no signs of microspheres falling off from the mold during handling.

Figure 7.94: Illustration of the RP component (A) Computer Aided Design (CAD) drawing of RP mold, (B) Transparent view of CAD drawing showing the internal interconnected channels, (C) 3D view of fabricated RP mold, (D) Top View of fabricated RP mold, (E) 3D view of LDPE mold, (F) Top view of LDPE mold

Figure 7.95: A) struts of the LDPE mold designed at 600 μm , B) channels of the actual gelatin scaffold

Figure 7.96: Inverse mold (LDPE mold filled with polystyrene microspheres)

7.1.2 The Procedure Concerning Mold Removal and Crosslinking

The mold removal process dissolves both the LDPE mold and the polystyrene microspheres. In the conventional particulate leaching process in which the particles are just touching each other and can be subjected to natural dispersion within the polymer solution, there exists a high possibility of particles being isolated within the scaffold structure that cannot be removed during the leaching process. The sintering

process used in this work creates a neck area that ‘fuses’ the contacting microspheres together. Furthermore, every microspheres are in contact with at least one microsphere underneath them due to gravity during the sintering process. Thus, the possibility of a microsphere becoming isolated from the rest and remaining within the scaffold structure is minimal. Having frequent changes of the solvent with slight agitation during soaking of the scaffold in the solvent also helps in the removal of the dissolved polystyrene out of the scaffold pores.

Scaffolds were fabricated using both types of inverse molds. From experimental observation, the scaffolds that did not undergo the dehydrothermal crosslinking process (see method 1 of Table 6.2) will swell and dissolve within a day when immersed in water due to the hydrophilic nature of the gelatin material. In contrast, crosslinked scaffolds were stable in water for up to 4 weeks.

The time taken for the crosslinking treatment is an important consideration in the inverse fabrication process as it relates to the preservation of the overall scaffold structure. In this research, two approaches were investigated (see methods 2 and 3 of Table 6.2). For the first approach, the dried gelatin infiltrated mold was immediately transferred to a vacuum oven for dehydrothermal crosslinking at 100°C. After 48 hours, the crosslinked construct was processed with heated toluene to dissolve the inverse mold and subsequently dried. The procedure of second approach is similar except that the processes are reversed: the mold is processed with heated toluene to dissolve the mold before the attempting the crosslinking treatment.

7.1.2.1 Crosslinking Treatment before Mold Removal

From the SEM observations, the pore structure of the gelatin scaffolds using the two approaches appear to be different from one another. For scaffolds that were fabricated using inverse molds without struts (microsphere template) and subjected to crosslinking treatment first (see Figure 7.4), the resulting pore structure consists of homogenously distributed spherical pores. In addition, the pores sizes appear to be uniform (around 85 μm) and most are observed to be interconnected to one another in

an ordered hexagonal arrangement. This demonstrates the usefulness of employing ultrasound agitation to align the microspheres into an ordered, close-packed position, which ultimately translates to an ordered array of pores.

Figure 7.97: Pore structure of scaffold fabricated using an inverse mold without struts.

A) X 30, B) X 100

For scaffolds fabricated using an inverse mold consisting of struts, the channels within the scaffold are visible (see Figure 7.5a). The SEM image of the pore structure in Figure 7.5b shows regions of highly organized, hexagonal arrangement of spherical pores as well as an interconnected network of channels. This demonstrates the effectiveness in preserving the overall structure of the scaffold by employing the crosslinking treatment before the mold removal process. It is important to note that

the process of sectioning the scaffold using a blade resulted in the collapse of the pore structure at some regions, especially those near the channels (see Figure 7.5b).

Figure 7.98: Gelatin scaffold fabricated via the inverse fabrication process. (A) gelatin scaffold immersed in ethanol, (B) SEM image of scaffold pore structure

7.1.2.2 Crosslinking Treatment after Mold Removal

The porous structure of the scaffolds that were subjected to crosslinking treatment after the mold removal process consist of pores that are non-spherical at some regions compared to those subjected to crosslinking treatment first (see Figure 7.6). The gelatin infiltrated molds are processed with heated toluene immediately after being freeze dried. Therefore, an additional drying step is required to remove the toluene from the scaffold before the crosslinking process. The drying process was carried out in an oven at 60°C to evaporate the solvent. Thus, the collapse of the scaffold structure may be attributed to the surface tension effects that are present in this additional drying step.

Figure 7.99: Gelatin scaffolds that were crosslinked after mold removal with regions of non-spherical pores (see circled area)

Another reason may be due to the fact that crosslinking is carried out only after the mold removal process. In this scenario, the yet-to-be crosslinked gelatin material in the inverse mold is still mechanically weak. Upon the dissolution of the inverse mold, individual pores begin to lose their shape due to the loss of the mechanical support previously provided by the microspheres (see Figure 7.7). On the other hand, scaffolds that were subjected to crosslinking before the removal of the mold are

observed to be mechanically stronger and are able to maintain their shape and structure upon the dissolution of the mold. Furthermore, the structural collapse might also be due to the inherent mechanical strength of the gelatin material itself. This is further aggravated by the highly porous nature of the scaffold in which its resulting mechanical strength may be insufficient to withstand the weight of the scaffold at the channel regions. The mechanical strength of a scaffold is inversely proportional to its porosity as demonstrated in the work of Sudarmadji *et al* in which a linear relationship was shown. Thus, a careful scaffold design process should take place to have a final scaffold with the desired properties for its required function. By using mechanically stronger scaffold materials e.g., PLA, PGA, issues related to structural collapse may be alleviated or avoided, though it still requires experimental work to verify that they are less susceptible to surface tension effects as in the case of biological materials.

Figure 7.100: Illustration on the importance of crosslinking treatment before the mold removal process

From these collective observations, one can conclude that the crosslinking process is a vital step in the inverse scaffold fabrication method. Not only does it preserve the pore structure and shape of the scaffold, it also stabilizes the scaffold in aqueous solutions, preventing them from dissolving too early. This is an important requirement of a TE scaffold as it must provide a temporary support structure for cell growth and

tissue development. Thus, the scaffold must be able to maintain their structural integrity for a period of time before a sufficient tissue mass is developed.

7.1.3 Scaffold Drying

Similar to the crosslinking treatment, the scaffold drying process is also an essential aspect of the scaffold fabrication procedure as the scaffolds must be dry in order to conduct the necessary experiments. In this research, three drying methods were attempted: 1) Freeze drying, 2) oven drying, and 3) critical-point drying (CPD).

For scaffolds dried using freeze drying and oven drying, the scaffolds appeared to be partially flattened. This suggests that the pore structure has already collapsed during the drying process. Furthermore, the scaffold pores appears to have lost its shape (see Figure 7.8a). On the other hand, the scaffolds that were dried using CPD retained their scaffold shape and pore structure (see Figure 7.8b). The primary causes of such structural deformation are the effects of surface tension. The scaffold specimen is subject to considerable forces, which are present at the phase boundary as the liquid evaporates. During the evaporation process, the surface tension at the solid-liquid interface pulls against any structures that the liquid is attached to. Delicate structures, e.g., highly porous scaffolds and cell walls that are mechanically weak tend to be broken apart by this surface tension as the solid-liquid interface moves by. Water, ethanol and several other solvents have high surface tension. This can be reduced by substituting the liquid with one of a lower surface tension, thereby reducing the damage during air-drying.

Figure 7.101: Pore structure of gelatin scaffolds that were dried using A) freeze drying, B) critical point drying

In freeze-drying, the process goes from the solid to gas boundary on the phase diagram (see Figure 7.9). However, some delicate structures are disrupted even by the solid-gas boundary. CPD, on the other hand, goes around the line to the right, on the high-temperature, high-pressure side. This route from liquid to gas does not cross any phase boundary, instead passing through the supercritical region, where the distinction between gas and liquid ceases to apply, thus eliminating surface tension effects. Carbon dioxide is one of the most commonly used supercritical fluid for CPD.

Figure 7.102: Critical point drying (blue arrow) goes beyond the supercritical point of the working fluid in order to avoid the direct liquid-gas transition seen in oven drying (green arrow) and also the solid-gas transition (red arrow) in freeze drying

7.1.4 Effect of Ultrasound Agitation on Pore Structure

The use of ultrasound agitation to assemble the microspheres into their closest packed position resulted in scaffolds with uniform pores that are aligned in a hexagonal arrangement. In contrast, scaffolds fabricated from inverse molds prepared without ultrasound agitation consist of disordered arrays of pores with no specific alignment and random interconnectivity (see Figure 7.10).

Figure 7.103: Pore structure of scaffold fabricated via inverse molds prepared without ultrasound agitation.

7.1.5 Summary of the Inverse Scaffold Fabrication Method

Based on the experimental observations, the overall procedure for the inverse scaffold fabrication approach is outlined (see Table 7.1). These procedures are planned according to the priority of the fabrication process, which is the preservation of the overall scaffold structure. Thus, the crosslinking process must be done before mold removal step in order to prevent the collapse of the pore structure. With regards to scaffold drying, the gelatin infiltrated mold can be dried using freeze drying method as the inverse mold is still present. The physical support provided by the mold is able to withstand the collapse of the gelatin scaffold structure. Any other drying process after this step must be carried out using the critical point drying method so as to avoid any deformation and collapse of the pore structure.

Table 7.7: Inverse scaffold fabrication procedure

Step	Description	Remarks
1	CAD design of RP mold	Using ProE, solidworks etc.
2	RP fabrication of mold	Using Objet with Fullcure 720 as model material
3	LDPE material casting	In vacuum environment at 130°C overnight
4	Removal of RP mold	Using Dynasolve 2000 at 60°C
5	Casting of polystyrene material into LDPE mold	Using a feeding rate of 50 μ l of IPA at 1 minute intervals
6	Microsphere assembly	Via ultrasound agitation
7	Mold sintering at 110 °C	According to desired time interval
8	Scaffold material casting	Using 3% gelatin solution containing 40% ethanol
9	Free drying of gelatin-infiltrated mold	To remove the water content
10	Dehydrothermal crosslinking of mold	Carried out at 100°C for 48 hours in vacuum oven
11	Mold removal	Using heated toluene
12	Scaffold rinsing to remove residual toluene	Rinsing with 100% ethanol
13	Critical point drying with liquid CO ₂	To obtain the final dry scaffold

7.2 Scaffold Characterization

This section discusses the results obtained from scaffold characterization studies in terms of pore interconnection size, local and global porosity.

7.2.1 Pore Interconnection Size

During the sintering process, the fusion between microspheres creates ‘necks’ which correspond to the interconnection between individual scaffold pores. The interconnection size will ultimately depend on the size of the neck formed between a pair of contacting microspheres. From SEM observations, a co-relation between sintering time and the interconnection size between scaffold pores can be observed (see Figure 7.11). The overall interconnection size increases at longer sintering intervals. This is to be expected as longer sintering times produce a larger degree of necking between microspheres. This corresponds to a larger neck radius and ultimately, a bigger interconnection size between the resulting pores.

Figure 7.104: Pore morphology of gelatin scaffolds that were created from inverse molds sintered at A) 1 hr, B) 8 hrs, C) 16 hrs, D) 24 hrs, E) 32 hrs, F) 40 hrs

The gelatin scaffold produced from the inverse mold that was sintered for 48 hrs was not shown in the figure. At this sintering duration, the scaffold produced after the mold removal and critical point drying process was too soft and crumpled during sectioning. Large void spaces within the pore structure can also be observed visually during the sectioning process. Such an observation suggests that the gelatin solution had not fully infiltrated into the inverse mold during the casting process. This can be

attributed to the large degree of necking between the microspheres after being sintered for 48 hours. The spaces between the microspheres are too small and even closed up at some regions to allow infiltration of the gelatin solution (see Figure 7.12). Consequently, the gelatin material that managed to infiltrate through the small spaces of the inverse mold contributed to a highly porous but mechanically weak structure.

Figure 7.105: Microsphere structure sintered for 48 hours illustrating a high degree of necking with little or no space between individual microspheres (see insert for closer magnification)

7.2.2 Frenkel's Model Predictions

The interconnection sizes between the pores at different sintering times were measured using Smile View (SMV) software and are tabulated in Table 7.2. Again, experimental data for scaffolds fabricated via 48 hrs of mold sintering are not included due to reasons explained previously. The tabulated data was drawn into a graph by plotting interconnection size against sintering time as shown in Figure 7.13.

Table 7.8: Co-relation between sintering time and interconnection size

Sintering time(hrs)	Interconnection size (μm)
2	14.2 ± 4.38

8	20.4 ± 6.27
16	26.1 ± 3.91
24	29.9 ± 4.13
32	33.3 ± 5.52
40	35.3 ± 6.04
48	-

Figure 7.106: Graph of pore interconnection size plotted against sintering time

The trend line in the graph illustrates an important characteristic of general sintering models (Equation 5.3): the rate of neck growth decreases as time progresses and the neck radius increases most rapidly during the early phases of the sintering process. These experimental results were also compared with the theoretical predictions from Frenkel's model. For theoretical model predictions, viscosity and surface tension data are needed to calculate the neck radius, y . These two material properties are both temperature dependent. The change in surface tension, γ with respect to temperature is known to be minimal compared to that of viscosity, η , which decrease exponentially with decrease in temperature. For this research, as the same sintering temperature (110°C) was used for all experiments, a fixed value for both surface tension and viscosity were used in the theoretical models. A number of authors have reported

surface tension data for various hydrocarbon polymers in the literature. The surface tension of polystyrene obtained by direct measurement on polystyrene melts, as tabulated by Wu *et al* , ranges from 0.026 to 0.0407 N/m in the temperature range of 20 to 200°C. By extrapolation, the surface tension value for polystyrene at 110°C is 0.0334 N/m. The viscosity of polystyrene at a glass transition temperature of 95°C is in the order of 10^8 Pa.s (Pascal seconds) as reported by Kobayashi *et al* . In his study which experimented with polystyrene at various temperatures, a temperature of 110°C would give an approximated viscosity of 20 MPa.s. The theoretical results from Frenkel's model as well as the experimental data for necking radius (corresponding to the scaffold pore radius which is half the interconnection size) is plotted and compared in Figure 7.14.

Figure 7.107: Theoretical predictions from Frenkel's model vs experimental data

The theoretical predictions from Frenkel's model differ from experimental data in an increasing order as sintering time increases. One possible explanation for this difference may be due to the method of obtaining a viscosity value for polystyrene. As the viscosity was calculated from experimental data of polystyrene melts at 170°C to 210°C, which has far exceeded the glass transition point for polystyrene, the actual viscosity value at 120°C which is closer to glass transition may be much higher than the calculated value. A larger viscosity value will bring the predictions of Frenkel's model closer to the experimental data.

For correction purposes, the Frenkel's model was introduced with a correction factor for this research. As the difference between the calculated values and experimental data at each sintering time interval are growing at a decreasing rate, an exponential function was introduced as a correction factor. The function uses a fraction of the sintering time over 48 hours (where the microsphere structure was observed to be completely densified) and the power factor of the equation in Figure 7.3 as shown:

$$y = \left(\frac{3r\gamma}{2\eta} \right)^{0.5} \text{Exp} \left(- \left(1 + \frac{t}{172800} \right)^{0.3102} \right) \quad (\text{equation 7.3})$$

In this semi-empirical Frenkel's model, the exponential term:

$\text{Exp} \left(- \left(1 + \frac{t}{172800} \right)^{0.3102} \right)$ is the correction factor in which t is the sintering time. The

theoretical predictions using Frenkel's model with the correction factor is tabulated in Table 7.3 and plotted in Figure 7.15. The difference in dimensionless neck radius (or pore radius) y/r , also taken as the degree of necking, is no more than 2% between theoretical predictions and experimental data at all sintering time intervals. Thus, Frenkel's model when used with the correction factor will be able to accurately predict the neck radius (which corresponds to the scaffold pore radius) at a specific sintering time interval with an error of less than 2%.

Table 7.9: Calculated data of Frenkel's model with correction factor.

Sintering time (Hrs)	Interconnection size (μm)		Dimensionless pore radius (y/r)		%error
	Frenkel's model (with correction factor)	Experimental data	Frenkel's model (with correction factor)	Experimental data	
2	7.18	7.11	0.082	0.081	0.1
8	11.76	10.17	0.134	0.116	1.8
16	1.45	13.13	0.164	0.149	1.5
24	1.61	14.95	0.183	0.170	1.3
32	1.73	16.65	0.196	0.189	0.7
40	1.81	17.65	0.206	0.200	0.6

Figure 7.108: Plot showing the theoretical predictions from Frenkel's model with correction factor and experimental data

7.2.3 Scaffold Porosity

The local porosities of the gelatin scaffolds fabricated from the inverse mold (without struts) prepared at different sintering time intervals are shown in Figure 7.16. All scaffolds were measured to be above 55% in porosity. As predicted, the porosity of the scaffolds increased with sintering time. This occurred due to the densification process in which the microspheres became more densely packed and the spaces between the microspheres became smaller as sintering time progressed. This resulted in a decrease in the center to center distance, l_{cc} , which according to Equation 5.12, decreases the unit cell volume (see Table 7.4). With a smaller unit cell volume and a fixed microsphere volume, the amount of gelatin material required to fill up the unit cell becomes smaller, and hence a higher porosity. For microsphere templates sintered at 2, 8 and 16 hours, the increase in porosity does not appear to be statistically significant from each other (see p values in appendix B1). There is even a slight

decrease in porosity from the 8hrs to 16 hrs time interval. This may be due to the larger amount of stacking faults within the microsphere templates at this sintering interval which resulted in lower porosity values. For the sintering times at 24, 32 and 40 hours, there was a gradual increase in scaffold porosity. The porosities of the scaffold at 32 and 40 hrs sintering intervals are both significantly different from that of 2, 8 and 16 hours of sintering time ($p < 0.05$).

Figure 7.109: Experimental porosity plotted against sintering time (n=6)

Table 7.10: Tabulated data of l_{cc} and Unit cell volume

Sintering time (Hrs)	Center-center distance l_{cc} (μm)	Unit cell volume (m^3)
2	8.68	4.63×10^{-13}
8	8.48	4.32×10^{-13}
16	8.32	4.07×10^{-13}
24	8.20	3.90×10^{-13}
32	8.11	3.77×10^{-13}
40	8.04	3.67×10^{-13}

7.2.3.1 Global vs Local Porosity

The average global porosities of scaffolds that were fabricated using inverse molds were also compared with local porosities (see Figure 7.17). At 2 and 16 hrs sintering interval, the global porosities are significantly higher than that of scaffolds fabricated using microsphere templates ($p < 0.05$). This is to be expected as the interconnected channels that are integrated within the scaffold contribute to the porosity of the overall structure as well. The overall trend can be observed to be increasing with time, although a slight but not statistically significant decrease in global porosity can be seen at both 8 and 40 hours of sintering time (see appendix B2).

Figure 7.110: Comparison between experimental global and local porosity

It can also be observed that the improvement in porosity as a result of the channels is less significant at 8, 24, 32, 40 hours sintering interval. Compared to the values at 2 and 16 hours sintering interval, the average global porosities of scaffolds at 8, 24, 32 and 40 hours are much closer to the average local porosity values, especially at the 40 hours interval ($p = 0.899$). This can be explained in terms of local porosity. At high sintering intervals, the unit cell volume of each individual microsphere becomes smaller and the space available for the scaffold material to infiltrate becomes smaller as well (see Figure 5.11). This would also imply that the volume of scaffold material in between individual pores is much smaller compared to those prepared at low sintering intervals. Thus, with a smaller volume of scaffold material in between every scaffold pores, the presence of a channel network, if placed within the scaffold

structure, would not remove much scaffold material compared to one which was prepared at low sintering intervals (see Figure 7.18).

Figure 7.111: Illustration on the amount of scaffold material taken up by a channel.
will be not improved by a considerable amount if prepared from inverse molds
fabricated at long sintering times

7.2.3.2 Local Porosity: The Effect of Ultrasound Agitation

Porosities were also compared between gelatin scaffolds fabricated via microsphere templates that have been prepared with and without ultrasound agitation (see Figure 7.19). The average porosity of those prepared with ultrasound agitation are greater compared to those without ultrasound agitation at all 3 measured time intervals and porosities are significantly greater at 24 hrs ($p < 0.05$). This is attributed to the effective method of microsphere arrangement using ultrasound. Microspheres that are assembled without ultrasound will consist of randomly aligned microspheres with extra air spaces within the structure (see Figure 7.20). In contrast, the use of ultrasound agitation is able to utilize this extra space by forcing/inducing the microspheres into their closest packed arrangement, which is the HCP arrangement (see Figure 7.21). Thus, the use of ultrasound agitation in preparing both the inverse mold and the microsphere template can be an effective way of improving scaffold porosity, which is desired for tissue engineering applications.

Figure 7.112: Porosity comparison between scaffolds fabricated from microsphere templates with and without ultrasound agitation (n=6)

Figure 7.113: Microsphere template assembled A) with ultrasound agitation and, B) without ultrasound agitation

Figure 7.114: Hexagonal arrangement of 88 μm polystyrene microspheres

7.2.4 Porosity Model Predictions with Compensation

The experimental results for porosity were also compared with theoretical analyses described in Chapter 5. Without porosity compensation, the gelatin scaffold porosities at all sintering time intervals are above 0.74, which is theoretically the closest packed density (and hence the maximum scaffold porosity) at sintering time $t = 0$. In practice however, perfect stacking is difficult to achieve and defects are common in microsphere assembled structures. Thus, to obtain the actual porosity of the gelatin scaffolds, porosity compensation was introduced in chapter 5 via two methods: 1) compensation from non-contacting points of a microsphere surface and 2) from stacking faults.

7.2.4.1 Compensation via Non-Contacting Points

From Figure 7.22, the first method of compensation resulted in a decrease in theoretical porosity as time increases and this decrease (from the non-compensated theoretical porosity) becomes larger with longer sintering time. From observations of the experiments, the number of necks formed by an individual microsphere increases

with sintering time due to densification and thus, there will be less non-contacting points to compensate. As such, one would assume that the compensation caused by non-contacting points would become less significant as time progresses. However, the decrease in unit cell volume at longer sintering times may outweigh the compensation caused by non-contacting points (see Equation 5.16 and Table 7.5). This means that the volume to compensate for the unit cell becomes larger for one non-contacting point and thus explains the effectiveness of the first method of compensation at longer sintering times (8 hours and more).

Figure 7.115: Comparison of experimental porosity with theoretical predictions

7.2.4.2 Compensation via Stacking Defects

From Figure 7.22, the second method of compensation brings the theoretical porosity way below their non-compensated theoretical values at all sintering times. P_{comp} , the volume of extra space caused by stacking defects was obtained by subtracting the porosity measured from scaffolds fabricated at 1 hr of sintering time (see Section 5.2.3.1) from 0.74.

Table 7.11: Porosity compensation via non-contact points

Sintering	No. of	Unit cell	volume (m^3) to	Total volume (m^3)
-----------	--------	-----------	---------------------	------------------------

time (Hrs)	necks	volume (m ³)	compensate for one non-contacting point.	to compensate
2	5.42	4.63 X 10 ⁻¹³	1.59 X 10 ⁻¹⁵	1.05 X 10 ⁻¹⁴
8	5.78	4.32 X 10 ⁻¹³	4.17 X 10 ⁻¹⁵	2.60 X 10 ⁻¹⁴
16	6.70	4.07 X 10 ⁻¹³	6.27 X 10 ⁻¹⁵	3.32 X 10 ⁻¹⁴
24	7.3	3.90 X 10 ⁻¹³	7.69 X 10 ⁻¹⁵	3.61 X 10 ⁻¹⁴
32	8.22	3.77 X 10 ⁻¹³	8.75 X 10 ⁻¹⁵	3.31 X 10 ⁻¹⁴
40	9.14	3.67 X 10 ⁻¹³	9.58 X 10 ⁻¹⁵	2.60 X 10 ⁻¹⁴

Although it is not obvious, the effectiveness of this method of compensation decreases slightly over time. This is due to the decrease in the volume of the extra space caused by stacking defects as time progresses. The decrease in volume of this extra space is calculated using the shrinkage of the overall bulk scaffold volume

$\left(\frac{l_{cc}}{l_{cc \max}}\right)^3$ at the desired time interval and is tabulated in table 7.6

Table 7.12: Porosity compensation via stacking defects

Sintering time (hrs)	% of original scaffold volume after shrinkage $\left(\frac{l_{cc}}{l_{cc \max}}\right)^3$	Total volume (m ³) to compensate $P_{comp} = 0.112$
2	96	5.18 X 10 ⁻¹⁴
8	89.6	4.83 X 10 ⁻¹⁴
16	84.4	4.55 X 10 ⁻¹⁴
24	80.9	4.36 X 10 ⁻¹⁴
32	78.2	4.22 X 10 ⁻¹⁴
40	76.2	4.11 X 10 ⁻¹⁴

Combining the two methods of porosity compensation (Equation 5.17), the theoretical values are observed to be much closer to the experimental values for local porosity (see t values in appendix B4). At the sintering intervals of 8, 24, 32 and 40 hours, the experimental porosities are not significantly different from that of the theoretical values obtained via the combined methods of compensation ($t < 2.57058$ for $\alpha =$

0.05). Thus, the porosity compensation can be said to be more effective at longer sintering times.

For global porosity, the theoretical predictions using equation 5.18 were compared with experimental results and are shown in Figure 7.23. The P_s values used in the equation were local porosity values that have been derived by the combination of the two porosity compensation methods. At $V_c = 3.04 \times 10^{-9} \text{ m}^3$, which is the total volume of the channel network calculated from PRO-E, the theoretical predictions derived from Equation 5.18 are within the standard deviation from experimental data ($t < 2.57058$ for $\alpha = 0.05$). As with the discussion earlier concerning the improvement in porosity (due to the existence of the channels) as sintering time increase, the theoretical predictions are in agreement with experimental data as well.

Figure 7.116: Comparison of theoretical global porosity values and experimental data. At longer sintering times, the global porosity becomes closer to local porosity values and the improvement in the porosity as a result of the channels becomes less effective (see Table 7.7)

Table 7.13: Comparison of theoretical local porosity and global porosity

Sintering time (Hrs)	Theoretical local porosity after compensation (%)	Theoretical global porosity (%)
2	64.6	65.8
8	67.9	69.0
16	71.7	72.6
24	74.8	75.6
32	78.3	78.9
40	82.3	82.9

7.3 Preliminary *In Vitro* Characterization

This section presents the results obtained from *in vitro* characterization studies concerning toxicity evaluation and cell proliferation on the fabricated scaffolds.

7.3.1 Scaffold Cytotoxicity Test

Cytotoxicity tests are useful in evaluating the toxicity derived from medical devices. A negative result suggests that the device is free of harmful extractable substances or has a quantity that is insufficient to cause acute reactions with cells. On the other hand, a positive test result implies that the device contains harmful substances that could threaten its clinical viability. RP systems were originally intended for fabrication of engineering models rather than bio-functional structures. In the present work, multiple steps and materials were involve in the gelatin scaffold fabrication process e.g., RP raw materials, polystyrene, LDPE as well as the solvents used to process the microspheres and to remove the molds. It is therefore important to assess the scaffold for any toxicity which may have been derived from these materials and processes.

The cell standard curve for the fibroblast cells is shown in Figure 7.24. The number of cells corresponds linearly with the fluorescence intensity with a relatively high correlation number (R^2). The line expression expressed on the graph will be used as a reference to calculate the approximate number of cells for the cell proliferation experiments.

Figure 7.117: Cell calibration curve for fibroblasts

The number of viable cells in all the well-plates, after exposure to scaffold extracts and the respective controls, was measured using Picogreen assay. The cell numbers were obtained from the calibration curve using the fluorescence intensity readings obtained from day 1 and day 3 and are plotted in Figure 7.25. In the case of the positive control, there were significant differences ($p < 0.05$) in the cell number as compared to the negative control on both observation days (see appendix for p values). This greatly reduced number of cells is a result of the toxic leachables derived from the positive control which could promote neither cell attachment nor proliferation. On the other hand, the fluid extracts derived from the gelatin scaffolds did not result in any significant differences in cell number ($p < 0.05$) as compared to fresh culture medium (negative control) under normal culturing conditions. Cell number has also increased in between the 2 observation days, suggesting that the gelatin scaffolds were non-toxic to the cells and were able to encourage the cells to proliferate.

Figure 7.118: Cytotoxicity assessment results: Number of fibroblasts cultured in fluid extracts derived from scaffolds relative to negative and positive controls (n=5)

Under microscopic observation, a confluent monolayer of cells with elongated morphology could be observed on the wells fed with the scaffold extracts and negative controls (see Figure 7.26a and c). Furthermore, these cells were stained negative using trypan blue (see Figure 7.26d and f). This demonstrates that the scaffolds were free from toxicity and are biocompatible to cells. On the other hand, the toxic effect of the positive control was evident: cells have completely detached from the well surfaces and appeared round in shape (see Figure 7.26b). The cells were not able to proliferate and no confluent monolayer can be observed. In addition, the blue stains observed from trypan blue staining are strong indications that extensive cell deaths have occurred. These findings are indicative of a cytotoxic response (see Figure 7.26e).

Figure 7.119: Microscopic images (20X) of cultured fibroblasts in 24-well plates and trypan blue staining respectively: (A, D) negative control, (B, E) positive control, (C, F) extracts derived from scaffolds

7.3.2 Scaffold proliferation test

To evaluate the cellular response of cells on 3D gelatin scaffolds fabricated using the inverse fabrication method, fibroblasts were seeded onto the scaffolds and cultivated for 14 days at 37°C in 5% CO_2 environment.

7.3.2.1 Cell Proliferation on Scaffolds prepared via Microsphere Templates

The number of cells on GS2, GS16 and GS32 (scaffolds prepared from the 1st type of inverse mold that were sintered at 2, 16 and 32 hours respectively) were first analyzed and compared at the 1st, 5th, 10th and 14th days of cultivation. Results presented in Figure 7.27 display a significantly higher average number of cells on GS16 and GS32 in comparison to GS2 on the 5th day of culture ($p < 0.05$). The number of cells on GS16 is higher than that on GS32, although not significantly, on both day 5 and 10 ($p = 0.279$ on day 5 and $p = 0.526$ on day 10). By the 14th day however, the number of cells on GS32 is the highest among the 3 types of scaffolds ($p < 0.05$). The high cell number on GS32 may be attributed to the larger interconnection size between scaffold pores which allows cells to easily migrate into the scaffold interior as compared to both GS2 and GS16. Furthermore, a scaffold with larger pore interconnection sizes is more accessible to nutrients from the scaffold interior. These collective factors allow cells to be more uniformly distributed across the scaffold pore structure and thus allowing more space for cell proliferation.

Figure 7.120: Cell proliferation on gelatin scaffolds (GS2, GS16, GS32) on the 1st, 5th, 10th, and 14th day of culture (n=5)

The average cell number on all scaffolds was observed to increase for all observation days, with the exception of GS2. For GS2, the cell number peaks at the 10th observation day after which the cell number decreases slightly. Although the decrease is insignificant ($p = 0.563$), this important observation suggests that the cells were unable to migrate into the scaffold interior due to the small interconnection size between pores. Consequently, cells that were unable to migrate through the pores remained in their location, congesting the scaffold periphery over time. As cells on

the surface of the scaffold started growing in multilayers, the cells in the interior were deprived of nutrients and thus, unable to proliferate further.

7.3.2.2 Results of Scaffolds Prepared from both types of Inverse Molds

Results obtained from gelatin scaffolds fabricated using the second type of inverse mold (GSC) as well as control scaffolds (CS) were compared with GS2, GS16 and GS32 in Figure 7.28. The number of cells on GSC significantly outgrew the cells on most scaffolds except GS16 on day 10 ($p < 0.05$). This can be attributed to the presence of the interconnected channel network which allows the surrounding culture medium to enter the scaffold through the channels. Thus, the cells residing in the scaffold interior become more accessible to nutrients and continue to proliferate. By comparing GSC and GS16, although having a similar range of pore interconnection size (both being sintered for 16 hours to fuse the microspheres), the presence of interconnected channels contributed to a significantly higher number of cells on GSC on observation day 14 ($p < 0.05$).

Figure 7.121: Cell proliferation on gelatin scaffolds (GS2, GS16, GS32, GSC, CS) on the 1st, 5th, 10th, and 14th day of culture (n=5)

For the control scaffolds (CS), the average cell number is lower compared to GS16, GS32 and GSC on observation day 5, 10 and 14. In fact, it can be observed that the number of cells has probably reached a plateau from day 10 with the cell number not significantly different from that of day 14 ($p = 0.675$). This observation can be explained in terms of its pore structure. As controls scaffolds were fabricated using the conventional casting/freeze drying approach, the pore structure was non-uniform and consisted of random pore interconnectivity as well as inconsistent pore sizes (see Figure 7.29). These are factors that can deter cells from infiltrating into the scaffold interior. Furthermore, the periphery of the scaffold is surrounded by a skin layer at some regions. This is a layer comprising of very small pores that are densely packed together and is a commonly observed feature at the periphery of freeze-dried scaffolds. Consequently, cells will be unable to infiltrate into the scaffold interior even from the surface due to the presence of this skin layer. The seeded cells are therefore limited to the surface on which they grow and form multi-layers, which is

almost akin to a 2D culture. Lastly, the large standard deviation on day 10 and 14 reflects the random pore structure of the gelatin scaffolds fabricated using conventional methods. With inconsistent pore sizes with random pore interconnectivity, cells will only be able to migrate and proliferate only at certain regions i.e., regions with pores that are large enough to provide space for cell accommodation and pores with interconnectivity large enough to allow cells to infiltrate into the scaffold. These regions are randomly localized within the scaffold due to insufficient control of the fabrication process over the pore structure.

Figure 7.122: The pore structure of control scaffolds showing irregular pore structure

Overall, the average cell number on GS32 remains the highest at observation day 14. On this day, the cell number on GSC, although not a significant difference (see appendix B5 for p values), is observed to be lower than that of GS32. GS32 was fabricated using microsphere templates prepared with a sintering time of 32 hours. Thus, although the 3D interconnected channels contributed to nutrient delivery as well as the global porosity of the overall scaffold structure, the cells may be unable to move through the pores (local regions) due to the smaller pore interconnection as compared to GS32. This could also be attributed to the distortion of the scaffold caused by cells, which was observed in the scaffolds during the culture period. Scaffold distortion is a common phenomenon observed on cell seeded collagen-based scaffolds. Due to its low mechanical strength, the scaffold material is unable to

withstand the tractional forces caused by the cells during motion. As cells attempt to attach themselves onto the scaffold, they spread and migrate from pore to pore. This constant spreading and migration causes the surfaces on which the cells attached to shorten/distorts in response as the cell attempts to migrate and spread itself after attachment onto the scaffold. The combination of traction forces caused by several hundreds of thousands of cells is sufficient to cause severe distortion on the gelatin scaffold. This distortion process partially closes up the channels, thus limiting the nutrient supply into the scaffold interior. To incorporate these channels in the scaffold while at the same time not allowing the channels to close up, further improvements on the design of scaffold will be necessary.

Chapter 8 – CONCLUSIONS AND FUTURE WORK

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

8.1 Conclusions

This research has focused on the development of an inverse scaffold fabrication approach that allows flexibility in controlling both architectural parameters and macro-features. The concept behind this fabrication methodology is an inverse strategy in which the entire internal structure of the scaffold is shaped using a lost-mold technique. The mold has been fabricated by combining RP technique with the particulate leaching process. Thus, the mold represents the negative replica of the entire scaffold architecture. The key contributions to this research are as follows:

8.1.1 The Development of the Inverse Scaffold Fabrication Method

With the increasing number of studies demonstrating the profound influences of scaffold pore architecture on cell behavior and overall tissue growth, a scaffold fabrication method with sufficient architectural control becomes imperative. As discussed in Chapter 2, the difficulty in controlling the process of conventional fabrication methods and the limitations of RP techniques has motivated this research to develop a new fabrication approach that allows the tissue engineer to shape the entire scaffold pore structure using an inverse mold.

In this work, an inverse method of scaffold fabrication has been successfully developed for TE applications. By employing both RP technologies and particulate leaching techniques, an inverse mold has been created to produce highly porous gelatin scaffolds with a highly ordered array of pore sizes. The mold was assembled from two components: mono-dispersed polystyrene microspheres and the RP mold, which was fabricated via a liquid based RP system, Objet (Eden 350V). This

proposed inverse method of scaffold fabrication provides the opportunity to manipulate the morphology of the scaffold at different scale levels: namely the macroscopic scale, defined by the outer shape of the scaffold and also the interconnected channels which play important roles in mass and flow transport; and the micro-scale, defined by the pores from the size of microspheres.

The detailed fabrication process of the inverse mold has been outlined. The feasibility in producing an ordered 3D assembly of microspheres through the combination of ultrasound agitation, a suitable solvent (IPA) and a low microsphere feeding rate has also been verified. Through the use of ultrasound agitation, the polystyrene microspheres were observed to be able to assemble into their closest packed arrangement in the RP mold. A HCP arrangement of the microspheres and the resulting scaffold pores were verified using SEM. By arranging the microspheres into their closest packed arrangement, scaffold porosity was observed to be larger as compared to scaffolds produced from molds that were prepared without ultrasound agitation. In addition, the process route from the inverse mold to the final scaffold has also been summarized. The sequence of dehydrothermal crosslinking and critical point drying is important in preventing the collapse of the scaffold pore structure.

Considering that the length scales of structures that govern several cell-specific functions such as adhesion, proliferation and differentiation are in the order of tens of microns, RP technologies alone or conventional fabrication methods will be unable to provide sufficient control over pore structure and hence, these functions. The proposed inverse fabrication method in this work has allowed the tissue engineer to have rigorous control over both the micro- and macro-scale features of the scaffold, allowing customization and realization of designer scaffolds in which specific structural requirements are necessary:

- a. The size of the microspheres determines the size of pores of the scaffold. Thus pore size can be tailored by changing the size of the microsphere size.
- b. Interconnected channels with complex configurations can be integrated within the scaffold using RP as long as it is able to be drawn using CAD programs.

- c. The shape of the RP mold also determines the shape of the scaffold. Thus the scaffold is not limited to simple geometries and complex shapes can be fabricated to fit well into the defect shape of the tissue/organ.
- d. The interconnection size between scaffold pores and the overall porosity of the scaffold varies according to sintering time. Hence, scaffolds with the desired porosity and pores with certain interconnection size can be fabricated by choosing the appropriate sintering time interval.

From this viewpoint, the inverse fabrication method has been proven to be a promising alternative way to prepare scaffolds with tailored pore architectures that can satisfy specific structural requirements of the tissue. The ability to precisely control the size of pores and the interconnection size can be crucial in studying certain cell behavior and can also function as an investigative platform for research on cell-cell, cell-scaffold interactions.

8.1.2 Theoretical Modeling of Scaffold Structure

A theoretical model of the sintering process has been developed so as to provide an insight to the scaffold pore structure. The model was useful in the prediction of the interconnection size between pores when the sintering time interval is known. With an increased sintering time, the interconnection becomes larger. However, this increase in interconnection size gradually becomes smaller as sintering progresses. A correction factor was also introduced to Frenkel's equation to bring the predictions closer to experimental values.

In addition, the porosity of the fabricated gelatin scaffolds with and without channels were modeled under the assumption of perfect stacking. Two types of porosity compensation were also introduced to account for the discrepancy in theoretical and experimental values. By combining both compensation due to stacking defects and non-contacting points of a microsphere, the theoretical predictions of local porosity and global porosity becomes closer to those derived from experimental measurements. This model serves as a basis for tissue engineers to tailor their scaffold porosity and interconnection size based on sintering time.

8.1.3 Preliminary *in vitro* Characterization

The *in vitro* response of cells to the fabricated gelatin scaffolds has also been preliminarily investigated. The presence of an interconnected network of channels within the scaffold visibly resulted in an improved cell proliferation as compared to gelatin scaffolds without channels that were prepared at 2 and 16 hours of sintering time. In addition, gelatin scaffolds that were prepared at 32 hours of sintering time showed the highest proliferation at the end of 14 days among the group of scaffolds without channels. This was probably due to the larger interconnection size between pores which allows the cells to easily infiltrate into the scaffold and also being more accessible to nutrients. On the basis of the cytotoxicity assessment results, it was clear that the multiple steps and materials involved in the fabrication process did not induce any toxicity in the gelatin scaffolds, and thus is suitable for tissue engineering applications.

8.2 Future Work

8.2.1 Improving the Design of the Inverse Mold.

Although the scaffold is observed to be comprised of hexagonally aligned pores due to the close packed arrangement of the microspheres, there still exist regions of randomly aligned pores. This arises from the natural stacking defects of the microspheres. Compared to the regions which are aligned in a HCP order, these regions may form a barrier of scaffold material in which the pores are less interconnected to one another, therefore preventing the infiltration of cells and the ingrowth of tissues at these regions. Thus, a method that is capable of precise control over the placement of individual microspheres can minimize or eliminate such stacking defects. This work can be expanded to explore methods or develop devices to control the placement of the microspheres within the RP mold. This however, may require costly machinery and time consuming back and forth testing. In addition, it may be necessary to devise practical experimental procedures to ascertain whether or not the mold has been completely filled. This is due to the possibility of the microspheres not filling up the mold at some regions due to the presence of features

e.g., ridge or ceiling within the mold which may hinder the movement of the microspheres into the mold during ultrasound agitation. Thus, a method that is capable of quantifying or visualizing the overall completeness of the mold filling would be beneficial to the end user in the mold design process.

In addition, more complex designs of the inverse mold can be explored to engineer other tissues. For example, in the case of the esophagus, a multilayered esophageal patch with a layer of small pore sizes for the epithelial layer and a layer with larger pore sizes and channels for the muscularis externa layer can be fabricated. The inverse mold can be designed such that it consists of a thin layer of smaller sized microspheres (for the epithelial layer) and another layer consisting of large size microspheres and channels for the muscle layer. Another example is bone tissues in which a porosity gradient can exist in the radial direction. In this case, a disc-shaped inverse mold consisting of channels and monodispersed microspheres can be sintered first, before adding an outer concentric layer of similar sized microspheres but sintered at a different duration.

Figure 8.123: Example of mold design for the multilayered esophageal tissue

8.2.2 Further Characterization on Scaffolds produced via Inverse Fabrication

Method

The characterization work done in this study has focused on co-relating sintering time interval to pore interconnection size and scaffold porosity. As scaffolds can have different porosity and interconnection size depending on the sintering interval, the study can be expanded to further characterize the overall permeability and also the

mechanical properties of the scaffolds as a result of their pore structure. The permeability of a scaffold can influence the accessibility of nutrients from the surrounding culture medium to the scaffold interior and hence affect the overall survival of the seeded cells. Some load bearing tissues would also require the use of scaffolds with certain stiffness e.g., bone tissues, cartilage, etc. Thus, having this information can be beneficial to the tissue engineer as the tradeoff between scaffold porosity and mechanical strength is one of the important considerations in scaffold design.

Furthermore, scaffolds prepared from inverse molds of different channel sizes and microsphere sizes can be characterized. Aside from sintering time, the effect of sintering temperature on microsphere necking size can also be modeled and investigated.

8.2.3 In Depth *In Vitro* and *In Vivo* Studies

This work has emphasized on preliminary *in vitro* characterization such as cell proliferation as well as cytotoxicity assessment. Further *in vitro* studies on dynamic cell culture using bioreactors can be proposed to study the responses of cells in a flow environment. Cells that were seeded onto scaffolds can be subjected to medium flow at an appropriate flow rate that mimicked the conditions in native tissues to facilitate nutrients and waste transport. The mechanical forces resulting from fluid flow are known to influence cell activity and phenotype, as well as enhancing ECM production, which can be characterized in future studies.

In addition, with the ability to tailor pore sizes as well as channel configurations using the inverse fabrication method, cell behavior such as proliferation, differentiation, ECM production etc, in response to different pore sizes and channel networks can be investigated. These can be followed up with *in vivo* implantation studies, targeting issues like angiogenesis and tissue ingrowth in response to scaffold porosity, pore size and pore interconnectivity. In this direction, the knowledge regarding the responses of cells/tissues to the various aspects of scaffold pore structure can be broadened.

8.3 List of Publications

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

8.3.1 Journal Papers

Published

1. Tan JY, Chua CK and Leong KF, Indirect fabrication of gelatin scaffolds using rapid prototyping technology. Virtual and Physical Prototyping (2010) Vol. 5 (1) p. 45-53.
2. Sudarmadji N, Tan JY, Leong KF, Chua CK and Loh YT Investigation of the mechanical properties and porosity relationships in SLS-sintered polyhedral for functionally graded scaffolds. Acta Biomaterialia (2011) Vol. 7 (2) p. 530-537.

Submitted

3. Tan JY, Chua CK, Leong KF and Chian, KS, Scaffolds for Esophageal Tissue Engineering: Design and Fabrication. Submitted to Biotechnology & Bioengineering.
4. Tan JY, Chua CK and Leong KF, An inverse approach to scaffold fabrication: combining microsphere leaching and Rapid Prototyping technology. Submitted to Acta Biomaterialia.

Papers under preparation

5. The PolyJet™ printing process of 3D fabrication: Fundamentals and modeling. To be submitted to Rapid Prototyping Journal.
6. Characterization of the physical structure of scaffolds fabricated from microsphere templates. To be submitted to Acta Biomaterialia.

8.3.2 Conference Papers

1. Tan JY, Chua CK and Leong KF, Indirect fabrication of tissue engineering scaffolds using rapid prototyping and a foaming process. Presented in The 4th International Conference on Advanced Research in Virtual and Rapid Prototyping, 6-10 October 2009, Leiria, Portugal.
Awards: Best paper for Biomanufacturing session

2. Tan JY, Liu A, Chua CK and Leong KF, Scaffold fabrication: an indirect approach combining microsphere leaching and Rapid Prototyping technology. Presented in 4th International PMI Conference, 15-17 September 2010, Ghent, Belgium.
Awards: PMI 2010 Innovation award.

References

Appendix

Plots of measured widths in various orientations

Tabulation of p values

p values for Figure7.16 (comparison of local Porosity at various sintering times)

Hrs	2	8	16	24	32	40
2		0.274151	0.420257	0.008641	0.002236	0.000143
8	0.274151		0.807415	0.063438	0.013536	0.001169
16	0.420257	0.807415		0.047752	0.010567	0.001064
24	0.008641	0.063438	0.047752		0.354662	0.060942
32	0.002236	0.013536	0.010567	0.354662		0.346884
40	0.000143	0.001169	0.001064	0.060942	0.346884	

p values for Figure7.17 (comparison of global Porosity at various sintering times)

Hrs	2	8	16	24	32	40
2		0.555599	0.257506	0.05576	0.002519	0.01038
8	0.555599		0.128065	0.014446	0.000694	0.003707
16	0.257506	0.128065		0.727978	0.107712	0.206115
24	0.05576	0.014446	0.727978		0.074008	0.209613
32	0.002519	0.000694	0.107712	0.074008		0.684271
40	0.01038	0.003707	0.206115	0.209613	0.684271	

p values for Figure7.17 (comparison between local and global porosity at specific sintering time)

Hrs	
2	0.017094
8	0.346693
16	0.035724
24	0.388618
32	0.228162
40	0.898509

p values for Figure7.19 (comparison for local porosity for scaffolds with and without ultrasound agitation)

Hrs	
8	0.078307
16	0.347439
24	0.037843

Tabulation of t values

t values for Figure 7.22 (Comparison of experimental local porosity and theoretical porosity with various compensation)

Hrs	Theoretical porosity after both compensations	Theoretical porosity with compensation from non-contacting points	Theoretical porosity with compensation from stacking faults	Theoretical porosity without any compensation
2	2.570376075	3.332198382	7.540802099	8.33978647
8	1.857867024	3.753375348	5.902962448	7.798470772
16	3.492167624	5.971968197	7.026725002	9.506525574
24	1.073705801	3.860515241	4.341873235	7.150398073
32	0.712394872	3.253640953	3.630987612	6.182251746
40	0.815572949	3.469640852	4.348800845	7.01116271

$t = 2.57058$ for $\alpha = 0.05$

t values for Figure 7.23 (Comparison of experimental global porosity and theoretical global porosity)

Hrs	Theoretical global porosity after both compensations
2	1.307557586
8	1.292460655
16	0.324353942
24	0.227307699
32	1.12329986
40	1.216348936

$t = 2.57058$ for $\alpha = 0.05$

Tabulation of p values for Cytotoxicity test (Figure 7.25)

	Negative control / scaffold extracts	Negative control / Positive control
Day 1	0.943223871	5.69027E-09
Day 3	0.315118704	2.34165E-10

Tabulation of p values for Figure 7.27 and 7.28

	GS2 1	GS16 1	GS32 1	GSC 1	CS 1	GS2 5	GS16 5	GS32 5	GSC 5	CS 5	GS2 10	GS16 10	GS32 10	GSC 10	CS 10	GS2 14	GS16 14	GS32 14	GSC 14	CS 14
GS2 1		0.666	0.1162	0.2946	0.06	2E-05	7.7E-06	3E-06	9E-07	0.003	6.4E-07	1.1E-07	3.7E-08	3E-08	1E-05	9E-06	5.86E-09	1.66E-08	3.1E-09	2E-04
GS16 1			0.1347	0.3721	0.04	2E-06	5E-06	1E-06	3E-07	0.003	3.9E-07	7.4E-08	1.8E-08	2E-08	1E-05	7E-06	2.81E-09	1.21E-08	1.7E-09	2E-04
GS32 1				0.3696	0.85	0.0017	6.7E-05	6E-05	2E-05	0.039	4.5E-06	5.6E-07	2.7E-07	2E-07	1E-04	4E-05	3.76E-08	5.14E-08	1.6E-08	8E-04
GSC 1					0.31	3E-05	1.2E-05	5E-06	1E-06	0.007	8.8E-07	1.4E-07	4.5E-08	4E-08	3E-04	1E-05	6.7E-09	1.91E-08	3.5E-09	3E-04
CS 1						7E-05	1.8E-05	8E-06	2E-06	0.017	1.1E-06	1.7E-07	5.1E-08	4E-08	3E-05	2E-05	7.21E-09	2.13E-08	3.7E-09	5E-04
GS2 5							0.00073	0.0007	1E-04	0.531	1.4E-05	9.2E-07	2.8E-07	2E-07	0.001	0.0002	2.44E-08	6.02E-08	1E-08	0.009
GS16 5								0.2794	0.7275	0.003	0.01785	0.00017	0.00014	2E-05	0.93	0.0991	4.96E-06	1.74E-06	9.1E-07	0.715
GS32 5									0.3272	0.005	0.00173	2.6E-05	1.5E-05	4E-06	0.349	0.0177	6.59E-07	4.84E-07	1.6E-07	0.293
GSC 5										0.002	0.0044	4.1E-05	2.3E-05	5E-06	0.821	0.0445	7.95E-07	5.83E-07	1.8E-07	0.548
CS 5											9.3E-05	5.9E-06	4.1E-06	1E-06	0.004	0.0006	4.21E-07	2.75E-07	1.3E-07	0.01
GS2 10												0.00497	0.00665	0.0003	0.019	0.5639	5.96E-05	9.2E-06	5.9E-06	0.144
GS16 10													0.53481	0.0725	2E-04	0.0052	0.014269	0.000257	0.00032	0.003
GS32 10														0.018	2E-04	0.0073	0.002552	8.15E-05	6.9E-05	0.004
GSC 10															3E-05	0.0005	0.417816	0.002252	0.00468	4E-04
CS 10																0.0958	6.61E-06	2.09E-06	1.2E-06	0.676
GS2 14																	0.000134	1.69E-05	1.5E-05	0.332
GS16 14																		0.003846	0.00904	2E-04
GS32 14																			0.26312	2E-05
GSC 14																				3E-05
CS 14																				