3D printing for biological applications

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2018

Ho, B. C. M. (2018). 3D printing for biological applications. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/74578>

<https://doi.org/10.32657/10356/74578>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**3D PRINTING FOR BIOLOGICAL
APPLICATIONS**

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2017

3D PRINTING FOR BIOLOGICAL APPLICATIONS

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A thesis submitted to the Nanyang Technological University in partial
fulfilment of the requirement for the degree of Doctor of Philosophy
2017

ACKNOWLEDGEMENT

Firstly, I would like to thank Assistant Professor Yoon Yong-Jin for his support and guidance throughout my years of graduate studies, providing me the chance to do my PhD while giving me the freedom to explore different areas of research.

I also would like to thank Dr Gary Ng from SIMTECH and Dr. Abhinay Mishra for providing me with their valuable advice and support for all my research.

I would also like to thank Assistant Professor Kimberly Kline the hospitality provided in her laboratory and allowing me access to their resources whenever needed.

I would like to thank all the students that I mentored, for assisting me through my PhD journey letting me pass down some “useful knowledge” to them.

And last but not least, I would like to thank Ms Yvonne Ang for all the support throughout my PhD especially for keeping me company as I write my papers.

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ABSTRACT

Different 3D printing methods with different biomaterials to create structures for cell-cell interactions were discussed. In this research, three different printed structures were printed from three different 3D printing techniques for the studies of cell interactions. Biopolymer of PCL and its nanocomposites have been designed and fabricated using 3D bioprinter for cardiac tissue engineering. Mechanical, thermal and biological characterisation were done for the printed scaffolds with cardiac cells to determine how these properties affect cell growth. Biodegradation was also tested using *Pseudomonas* lipase to determine the possible use of these printed scaffolds for cardiac tissue engineering. Results showed that cardiac cells grow better on 3D printed scaffold of PCL with 1% CNT as compared to PCL and PCL with 3% CNT. Next, to have a better understanding on how bacteria cells communicate with one another in a liquid culture, PEGDA hydrogel was designed and fabricated using stereolithography system. Quorum sensing (QS) through secreted small molecules is one of the ways in which *Pseudomonas aeruginosa* cells communicate with one another. So, by spatially designing honey-combed structures, QS molecules can be diffused across the hydrogel for cells to interact. Mechanical, swelling and printability properties of the structures were determined to obtain fully functional structures for cell interactions for 24 hours. Well-defined honey combed structures could be printed with PEGDA with 1% Irga819. These structures will then be seeded with *P. aeruginosa* for further studies. Finally, two-photon polymerisation was used to print and spatially pattern bacteria cells in an encapsulated gelatin matrix. These structures save time as cells can be printed directly. A predatory interaction between *E. coli* and *S. aureus* was observed through a microcolony assay. Initial results from the distal and mix microcolony assay showed that this predatory effect was only observed when *E. coli* and *S. aureus* were in close proximity of each other. Predatory mechanism was deduced through RNA sequencing and Transposon screening. Due to its high resolution, 2PP was used to spatially pattern *E. coli* and *S. aureus* with distance up to 50 microns apart. With more research on 3D printing and biomaterials, the search for the “killer application” in biology might be realised in the near future.

ACKNOWLEDGEMENT.....	III
ABSTRACT.....	I
TABLE OF FIGURES.....	IV
CHAPTER 1: INTRODUCTION.....	7
1.1 BACKGROUND	7
1.2 OBJECTIVES.....	11
1.3 SCOPE.....	14
CHAPTER 2: LITERATURE REVIEW.....	15
2.1 3D PRINTING	15
2.1.1 What is 3D printing?	15
2.1.2 Advantages and disadvantages of 3D printing.....	16
2.1.3 Types of 3D printing mainly used for biology.....	16
2.2 APPLICATIONS OF DIFFERENT 3D PRINTING TECHNOLOGIES.....	22
2.2.1 Bioprinting	22
2.2.2 Stereolithography (UV based printing).....	25
2.2.3 Direct laser writing/Two photon polymerisation (NIR based printing).....	27
2.3 CURRENT ISSUES AND FUTURE APPLICATIONS.....	30
2.3.1 Biomaterials	31
2.3.2 Hardware	32
2.3.3 CAD software	33
2.3.4 Automation of pre-and post-processes.....	34
2.4 Final comments	34
CHAPTER 3: 3D PRINTED SCAFFOLD FOR CARDIAC TISSUE ENGINEERING	35
3.1 INTRODUCTION	35
3.2 MATERIALS AND METHODS	36
3.2.1 Materials	36
3.2.2 Nanocomposite Preparation for printing.....	37
3.2.3 3D printing of PCL and PCL-CNT scaffolds with RegenHU Bioprinter	37
3.2.4 Raman Spectroscopy	38
3.2.5 Thermal Characterisation.....	38
3.2.6 Mechanical characterisation.....	38
3.2.7. Electrical Conductivity.....	38
3.2.8 Enzyme Degradation.....	38
3.2.8. Biological compatibility	39
3.2.10 Cell imaging on Scaffold.....	39
3.2.11 Statistical analysis.....	40
3.3 RESULTS AND DISCUSSION	41
3.3.1 3D printing of scaffolds.....	41
3.3.2. Characterisation of 3D printed PCL nanocomposites	44
3.3.3. Thermal behaviour	45
3.3.4. Nanoindentation (mechanical response of scaffolds) and Conductivity.....	47
3.3.5 Enzymatic Biodegradation	49
3.3.6. Cytotoxicity assay and cell compatibility.....	51
3.4 CONCLUSION.....	54
CHAPTER 4: HONEYCOMBED STRUCUTRES FOR CONTAINMENT OF LIQUID BACTERIA CULUTRE FOR QS SENSING.....	55
4.1 INTRODUCTION	55
4.2 MATERIALS AND METHODS	57
4.2.1 Material for printing	57
4.2.2 Preparing solution for 3D Printing.....	57
4.2.3 Printing of Scaffold	57
4.2.4 Tensile Test.....	58

4.2.5 Swelling Test	58
4.2.6 Stereo Microscopy.....	59
4.2.7 Scanning Electron Microscopy (SEM).....	59
4.2.8 <i>Pseudomonas Aeruginosa</i> Culture & Observation	59
4.3 RESULTS AND DISCUSSION	60
4.3.1 Tensile Test for Dry Dog-boned samples.....	60
4.3.2 Tensile Test for Swelled Dog-boned Samples	62
4.3.3 Swelling Test	65
4.3.4 Stereo Microscopy Images	66
4.3.5 Scanning Electron Microscopy (SEM) Test	68
4.3.6 Summary of Results	70
4.3.7 <i>Pseudomonas Aeruginosa</i> Culture & Observation	71
4.4 CONCLUSION.....	72
CHAPTER 5: HYDROGEL STRUCTURES FOR STUDIES OF <i>E. COLI</i> AND <i>S. AUREUS</i>	
INTERACTIONS.....	73
5.1 INTRODUCTION	73
5.2 MATERIALS AND METHODS	74
5.2.1 Bacterial Strains and Growth Conditions	74
5.2.2 Macrocolony assay.....	75
5.2.3 RNA extraction, RNAseq and Analysis.....	75
5.2.4 Electrocompetent cells/Transposon Library Screen	76
5.2.5 Transposition mapping.....	77
5.2.6 Planktonic Growth Assays	77
5.2.7 3D printing of gelatin microstructures.....	77
5.2.8 Statistical analysis.....	78
5.3 RESULTS AND DISCUSSION	79
5.3.1 Proximal distal Microcolony assay.....	79
5.3.2 Macrocolony assay analysis between <i>E. coli</i> and <i>S. aureus</i>	80
5.3.3 Transposon screen and RNA sequencing results	83
5.3.4 2PP of Gelatin microstructures.....	87
5.4 CONCLUSION.....	88
CHAPTER 6: CONCLUSION & FUTURE WORK.....	89
REFERENCES.....	92
APPENDIX.....	103

TABLE OF FIGURES

Figure 1 (a) Epithelial and endothelial cells adhere to the basement membrane via only one surface, whereas mesenchymal cells, including fibroblasts, are surrounded by the extracellular matrix (ECM). In addition, the types of adhesive junctions vary in different cell types, with epithelial cells assembling hemidesmosomes. Picture taken from [5]. (b) Schematic highlighting the sites and types of polymicrobial diseases commonly located throughout the human body[13].	8
Figure 2 Number of publications on 3D printed structures for biological applications over the past five years. The data shown reflect the most recent data searched (31 Jan 2018) with Scopus. The following general search string was used: topic = (3D printing), and (Cell Biology) and published = (2013 to 2018).	11
Figure 3 Overall schematic diagram for the study on 3D printed PCL and its composites for cardiac tissue engineering	12
Figure 4 Overall schematic diagram for the study on 3D printed PEGDA scaffold for cell to cell interactions	13
Figure 5 Overall schematic diagram for the study on 3D printed Gelatin scaffold for microbiology	14
Figure 6 Brief schematic diagram of product development cycle for 3D printing. Picture taken from [35]	15
Figure 7 3D printing technologies based on the classification mentioned and method to form the structures. Figure taken from [35].	17
Figure 8 Bioprinting of blood vessels. A) Bioprinter loaded with cells and support B) Printing and patterning of support (purple) and cell (orange) in filament form C) formation of blood vessels. Picture adapted from [68].	23
Figure 9 (Left) Schematic representation of bioprinting of agarose template fibres and subsequent formation of microchannels via template micromolding. A) A bioprinter equipped with a piston fitted inside a glass capillary aspirates the agarose. After gelation at 4 °C, agarose fibres are bioprinted at predefined locations. B) A hydrogel precursor is cast over the bioprinted mould and photocrosslinked. C) The template is removed from the surrounding photocrosslinked gel. D) Fully perfusable microchannels are formed. (Right) Photographs of the different bioprinted patterns. A-i) planar bifurcating pattern, B-i) 3D branching pattern and C-i) 3D lattice pattern. Green refers to the bioprinted template with agarose while red refers to microchannels perfused with a fluorescent microbead. Picture taken from [64]	24
Figure 10 (a) Schematic illustration of separation of captured bacteria by inertial focusing. (b) Illustration of Dean vortices in a channel with trapezoid cross-section. (c) Photograph of the 3D printed device. Picture taken from [52]. (d) Schematic illustrations of 3D immunomagnetic flow assay. The magnet–spacer assembly was placed in the opening of the HEMOS. Picture taken from [51].	26
Figure 11(a) Mechanism of TPA when simultaneous excitation. (b) and (c) Illustration of two methods for increasing the probability that TPA occurs: density of photon is increased by (b). spatial compression using objectives with high numerical aperture, (c). temporal compression using ultrafast lasers. Picture taken from [89]	27
Figure 12 Scanning electron microscopy images illustrating the fabrication of 3D composite polymer scaffolds by sequential DLW. a) In the first step, 3D frameworks consisting of protein-repellent PEG-DA with 4.8% PETTA are polymerised and developed. b) Frameworks are then cast with the photoresistOrmocomp and cubes (one cube is highlighted in red) are precisely attached to the PEG-DA beams in a second DLW step. c) Higher magnification image of anOrmocomp cube (highlighted in red). Picture taken from [94].	29

Figure 13 Microporous 3D cell culture scaffolds produced by direct laser writing (DLW). Scanning electron microscopy (SEM) images of 3D PETTA scaffolds with mesh sizes of 2 mm, 5 mm, and 10 mm fabricated by DLW. Picture taken from [95]	30
Figure 14 (a) Stereo microscopic images of PCL and its nanocomposites. (Scale Bar 2 mm) (b) Average line width of the grid and average distance between the strips of PCL and its nanocomposites w.r.t. percentage of CNT present in the sample (Inset figure showing the representative printed picture of PCL-CNT-5 scaffold.)	42
Figure 15 Optical images of single window and cross junction of 3D printed pure PCL and its indicated nanocomposite grids (a) PCL (b) PCL-CNT-1 (c) PCL-CNT-3 (d) PCL-CNT-5. (Scale Bar 400 μ m)	43
Figure 16 Raman spectra of pure MWCNT, PCL and its indicated nanocomposites	45
Figure 17 DSC thermograms of PCL and its indicated nanocomposites (a) heating curves (b) Graph representing the change heat of fusion (J/g) and melting temperature w.r.t. CNT percentage (c) cooling curves (d) Graph representing the change heat of crystallisation (J/g) and melting temperature w.r.t. CNT percentage in the sample.	46
Figure 18 Nanoindentation of PCL and its indicated nanocomposites (a) Representative Maximum Load vs. displacement graph (b) Bar graph representing the comparison of hardness, modulus and average load.....	48
Figure 19 (a) Percentage weight loss of PCL and its indicated nanocomposite scaffolds during enzymatic (lipase from <i>Pseudomonas</i> sp., Type XIII) degradation at 37 °C. (b) SEM images of PCL and its indicated nanocomposites before and after degradation. The '0h' and '6h' indicates the time before and after 6h degradation respectively.	50
Figure 20 (a) MTT assay of H9C2 myoblast cells in PCL and its indicated nanocomposite scaffolds. (b) Fluorescence images of H9C2 myoblast cells obtained through dye staining after 4 days of growth on PCL, PCL-CNT-1, and PCL-CNT-3 scaffolds. (Scale Bar 100 μ m)	52
Figure 21 Fluorescence images of live (green) and dead (red) H9C2 myoblast cells obtained through dye staining after 3 days of growth on (a) Control (b) PCL, (c) PCL-CNT-1, and (d) PCL-CNT-3 scaffolds. All the images are at a magnification of 10X.....	53
Figure 22 Tensile Stress-Strain Curve for Dry Samples. Black = 0.1% Igracure 819, Red = 0.5% Igracure 819 and Blue = 1% Igracure 819.	60
Figure 23 Tensile Modulus Chart for Dry Samples.....	61
Figure 24 Tensile Stress-Strain Curve for Swelled Samples	63
Figure 25 Swelling Ratio Bar Chart 0.1%, 0.5% and 1.0% Igracure concentration hydrogel scaffolds soaked in TSB medium and LB medium	65
Figure 26 Fractured Scaffold Due to TSB Swelling.....	66
Figure 27 Fabrication of 3D microstructures through 2-Photon direct laser writing	78
Figure 28 a) CFU count of PAO1, UTI89 & MN8 on the Distal microcolony assay after 48hours. b) CFU count of PAO1, UTI89 & MN8 on the Mixed microcolony assay after 48hours. N =3 Black bar = Individual species, Red bar = PAO1 & MN8 grown together, Blue bar = PAO1 & UTI89, Yellow bar = UTI89 & MN8 and Green Bar = All 3 species grown together	80
Figure 29 CFU count of MN8 at different timepoints. Dark Grey bar = Individual UTI89, Light Grey bar = UTI89 enumeration with MN8, Black bar = Individual MN8 species, Red bar = MN8 enumeration with UTI89	81
Figure 30 CFU count of different strains of <i>S. aureus</i> grown with UTI89 at 8hrs and 24 hrs. Black colour = MN8, Blue bar = ISP427, Red bar = RN450, Green bar = HG001 & Orange bar = RN1	82
Figure 31 CFU count of UTI89, UTI89 Δ TonB and MG155 grown with MN8 at 8 hrs and 24 hrs.....	82

Figure 32 Fluorescence intensity reading of HG001 when grown individual (red), Mix with UTI89(beige), MG1655 (red with black). When UTI89 and MG1655 was taken for fluorescence there is nothing.	84
Figure 33 Transcriptional gene expression profiling of <i>E. coli</i> in microcolonies assay with <i>S. aureus</i> . Differentially regulated genes and functional categories (≥ 2 -fold change, FDR < 0.05)	85
Figure 34 Transcriptional gene expression profiling of <i>S. aureus</i> in microcolony assay to <i>E. coli</i> . Differentially regulated genes and functional categories (≥ 2 -fold change, FDR < 0.05).	86
Figure 35 (a) Cylindrical Pattern containing <i>S. aureus</i> bacteria contained in Gelatin matrix (inset picture depicts the Confocal Image) (b) Cylindrical Pattern containing <i>E. coli</i> bacteria contained in Gelatin matrix (c) Interconnected Dumbbell pattern containing <i>S. aureus</i> and <i>E. coli</i> bacteria (inset picture depicts the Confocal Image).....	87

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Cell–cell interactions refer to the direct interactions between cells; and cell with the extracellular matrix. These interactions are crucial in the development and functioning of multicellular organisms as they coordinate and regulate cell signalling and functions. Therefore, a better understanding of cell–cell interactions is critical to many physiological and pathological processes, such as cell fate and differentiation, cancer metastasis, immunological interactions, and pathogenesis[1-4]. Cells are mainly regulated by physical and chemical cues. The fact that cells respond to their physical microenvironment is perhaps obvious[5, 6]. Microscale features which are comparable to the size of cells are present in the extracellular matrix (ECM) or the artificial scaffolds. They provide cell adhesion to the surface that typically results in whole-cell contact guidance. Contact guidance was first observed in 1911[7] and was described as the alignment of cells with topographical features[8]. For example, a different combination of internal and external mechanisms is essential for cell fate regulation whether pluripotent stem cells are grown *in vivo* or *in vitro*. Internal mechanisms are specific regulation factors produced by the cells themselves while external mechanisms are signals provided by the local niche microenvironment, including secreted growth factors or contact from other cells, and the ECM[6]. The local environment can be remodelled by the stem cells according to the signals they received[7, 9].

Cells are exposed to ECM proteins in the body[10] (Figure 1a). Epithelial and endothelial cells encounter the basal membrane which confers cell polarity but not with the apical or plasma membrane. For connective tissue, cells (fibroblasts in the dermis or chondrocytes in cartilage) are surrounded by the ECM, blood cells are also exposed to soluble ECM proteins such as fibronectin. Cells exploit several different cell surface receptors, one of which is integrins, that is for adhering to the ECM[11]. Mechanotransduction is an upcoming field where cells are subjected to mechanical cues such as forces and how cells respond through morphological changes or biological responses in terms of cell differentiation and proliferation[12].

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Figure 1 (a) Epithelial and endothelial cells adhere to the basement membrane via only one surface, whereas mesenchymal cells, including fibroblasts, are surrounded by the extracellular matrix (ECM). In addition, the types of adhesive junctions vary in different cell types, with epithelial cells assembling hemidesmosomes. Picture taken from [5]. (b) Schematic highlighting the sites and types of polymicrobial diseases commonly located throughout the human body[13].

In this research, microbes are used as the test subjects, simply because they are much easier to handle as compared to eukaryote cells and their metabolic, signalling and architectural networks can be broken down into modules for easy analysis and engineering. Microbes are rarely in single-species planktonic forms, but they often thrive in complex polymicrobial biofilm communities, attached to both living and non-living surfaces. Polymicrobial biofilm communities consist of a varied collection of microorganisms (fungi, bacteria, and viruses) encapsulated in a self- and/or host-derived hydrated polysaccharide matrix[14]. At any given point, an estimated 600 to 1,000 unique bacterial species are either permanently or transiently colonising human mucosal sites such as gastrointestinal (GI) tract, oral cavity and respiratory systems (Figure 1b)[15, 16]. Bacteria have evolved over thousands of years, expressing various species-specific physical and chemical interactions to survive with the large polymicrobial communities present in its local environment. Some microbes developed positive relationships to facilitate co-habitation in the environment or

metabolised by-products secreted from other species while others have developed predatory approaches during co-colonisation. These relationships are established by contact-dependent attachment or secretory products via small molecules, or a combination of the two events[17].

Multispecies communities were first observed on human tissues using the primitive microscopes [13]. However, an overall scope and understanding of how multi-species interactions affect the progression and severity of the human disease still remains a mystery[18]. Many studies on microbiology suggested that a single virulence factor or a single pathogen microbe was sufficient in causing a disease. While this is true for some human diseases, immunisation or drug treatment against single virulence factors or single microbe were proven to be unsuccessful. Virulence in some organisms are no longer caused by a single virulence factor, likewise, some diseases are no longer classified as a single species infection[19].

Culture-dependent isolation techniques lead to many diseases being characterised as being monomicrobial in nature. However, with the advancement of culture-independent community analysis methods, diseases such as oral infections, otitis media, diabetic foot wound infections and cystic fibrosis are now recognised as true polymicrobial infections. In fact, with the known composition of microbial populations, disease severity and outcomes can be predicted. Therefore, species/strains identification using next generation sequencing coupled with novel studies of polymicrobial relationships can lead to better prevention against potential diseases caused by microbes. In addition, new or improved therapeutic measures for combating polymicrobial diseases may be developed.

As biology is moving towards a quantitative era, a robust, biocompatible method for precision control in the spatial and temporal association for cells is needed to further aid the understanding on how cells interact and communicate with one another in their natural environment[20]. Although several methods such as optical, electrical, magnetic, hydrodynamic, and contact printing technologies are available for the patterning of cells, these methods still have their individual limitations[21-25]. Firstly, cell native state may be affected due to the external modifications. For example, magnetic assembly requires cells to be labelled with magnetic particles which affects certain cell pathways. Electrical methods require the use of a special non-conductive media which lacks essential nutrients that affects cell growth or physiology adversely[22]. Although, optical tweezers provide a label-free and contactless approach, cell

damage may occur due to the use of high laser power for cell manipulation[21]. Secondly, fabrication of high-precision technologies requires multiple expensive machinery and complex experimental setup for cell manipulation. Thirdly, high-throughput methods (magnetic assemblies) are not able to achieve single level cell precision. Finally, most of the existing technologies are unable to maintain cell assemblies in suspension, thus reducing the applications for the study of cell–cell and cell–matrix interactions[4].

As mentioned, most of the patterning cells techniques require multiple expensive equipment such as embossing, injection moulding, laser ablation, micro-machining and soft lithography[26]. Other disadvantages include big and bulky equipment needed, labour intensive, time consuming when changes are required in design and limited availability of biological materials. For small-scale confined structures production in a laboratory environment, soft lithography, a multiple step process, is the gold standard. As cell biologists may not have the time to learn the fabrication process[28], quick and easy fabrication of devices or simple structures are preferred[27]. Many researchers are looking to 3D printing as an alternative due to its advancements in terms of product resolution and fabrication speed. Publications on 3D printed structures for cell interaction studies have been on the rise in the last five years (Figure 2). Fabrication of unique structures using a range of biocompatible materials with high porosity, resolution and defined pore structure are some notable advantages associated with 3D printing over conventional methods. With the introduction of 3D Bioprinting, a wide range of biomaterials including living cells and proteins could be printed directly as well[29-32]. Recent advancement in 3D printing capabilities also allowed printing of micro and nano scale 3D features. Greater topographical flexibility can be obtained with multiple 2D layers stacked onto each other. This allows high-precision construction of a multi-layered transport/ micro-vascular channel network in a range of 100–300µm with an accuracy of tens of microns[29, 30, 33]. This could possibly aid in finding the “killer application” for 3D printing and lead to its acceptance by researchers, especially in the biological field.

Figure 2 Number of publications on 3D printed structures for biological applications over the past five years. The data shown reflect the most recent data searched (31 Jan 2018) with Scopus. The following general search string was used: topic = (3D printing), and (Cell Biology) and published = (2013 to 2018).

1.2 OBJECTIVES

Since most 3D printing machines are commercially available, the question that was of interest was which 3D printing technique is suitable for creating structures while confining cells in a specific environment for cell interaction studies. The main aim of this research was to demonstrate the different 3D printing techniques that could be applied to the field of biology especially for the fabrication of biocompatible structures that would allow the study of cell-cell interactions. In this research, three different types of machines were used to fabricate a confined environment for the study on interactions that cells have with materials and cell-cell interactions.

Firstly, the fabrication of well-defined scaffold structures constructed with polycaprolactone (PCL) and PCL-carbon nanotube (PCL-CNT) for cardiac tissue engineering were designed and printed using a bioprinter. To ensure that the 3D printed PCL or PCL-CNT scaffolds possessed similar or improved characteristics over traditional fabrication techniques, a characterisation study on thermal, mechanical and biological properties of the printed scaffolds with varying CNT percentages were performed. Functionalised CNTs and its interactions with the PCL matrix was

then observed using the Raman spectroscopy. Crystalline behaviour and mechanical properties of the composites was characterised using differential scanning calorimetry (DSC) and nanoindentation respectively. Since the scaffold can be used as an implant, biodegradation rate and degradation specificity on the scaffold was also carried out using *Pseudomonas* Lipase enzymatic media. Biocompatibility of the scaffold was done with H9c2 cells from rat origin using fluorescence imaging and MTT assay. Initial results showed 3D printed PCL and its CNT composites should be able to show potential for use in cardiac tissue engineering (Figure 3).

Figure 3 Overall schematic diagram for the study on 3D printed PCL and its composites for cardiac tissue engineering

Secondly, the fabrication of spatially confined chambers made from poly (ethylene glycol) diacrylate (PEGDA) for interaction studies between liquid bacteria communities was done using stereolithography. Honey-combed structures and dog-boned samples were designed using SOLIDWORKS software and printed using Asiga PICO2. Post-processing in the form of cleaning and post-curing was done to ensure removal of excess uncured resin. Printability of PEGDA with the different concentrations of photoinitiator Igracure819 was tested to obtain the optimal printing accuracy of structures. Once the printing conditions are optimised, mechanical (tensile testing), surface observation (SEM imaging) and swelling (swelling test) properties was characterised on

the 3D printed dog-boned structures. After the right concentration of photoinitiator was determined, honey-combed structures were printed and observed with fluorescence *P. aeruginosa* to see whether any cell-cell interactions (QS sensing) can be observed between the different species (Figure 4).

Figure 4 Overall schematic diagram for the study on 3D printed PEGDA scaffold for cell to cell interactions

Finally, the confinement of *E. coli* and *S. aureus* cultures in a semi-solid gelatin structure for bacteria interactions studies was done using microscopic three-dimensional (μ 3D) printing. A predatory effect was observed initially in a microcolony assay when *Escherichia coli* (*E. coli*) was mixed together with *Staphylococcus aureus* (*S. aureus*). To determine the killing mechanism of *E. coli* on *S. aureus*, RNA sequencing and transposon screening were performed. To observe the killing effect in the micro level, various 3D structures of biopolymer gelatin and initiator rose bengal with bacteria were fabricated via two-photon polymerisation (2PP). Concentrations of both gelatin and rose bengal (photoinitiator) need be determined in order to obtain well-defined structures. Biocompatible studies on entrapped bacteria were done with confocal microscope using fluorescence *E. coli* and *S. aureus*. They were kept in the confined environment for up to 8 hours (Figure 5).

Figure 5 Overall schematic diagram for the study on 3D printed Gelatin scaffold for microbiology

1.3 SCOPE

The scope of this research is to show the possible applications of the different printers for different biological applications especially for cell-cell interactions. First, the bioprinter was used to fabricate PCL and PCL-CNT grid scaffolds for cardiac tissue engineering. Next, to obtain higher resolution, PEDGA was used with SLA system to develop and improve current structures for liquid bacteria cell-cell interactions. Finally, to trap bacteria aggregation and pattern them, a control spatial environment multiphoton polymerisation (2pp) was used.

CHAPTER 2: LITERATURE REVIEW

2.1 3D PRINTING

2.1.1 What is 3D printing?

Three-Dimensional (3D) printing also known as additive manufacturing (AM) or rapid prototyping (RP) technology consists of several different automated fabrications techniques. The whole process is a combination of design modelling and production shown in Figure 6. Firstly, 3D models can either be designed by the user or obtained from computed tomography (CT) scans or laser scans of the 3D object. These models can then be edited or redesigned using available commercial software such as Rhino, Solidworks, Medical modeller etc. Some of the software may contain additional programmes for optimising the printing process. Once the models are ready, these models are converted to STL file or the new AMF format. After which it is sliced into a series of 2D cross-sectional layers, creating a path for the printer to trace/move from the bottom[34]. Depending on the 3D printing technology, post-processing may be required to obtain the finished product.

Figure 6 Brief schematic diagram of product development cycle for 3D printing. Picture taken from [35]

2.1.2 Advantages and disadvantages of 3D printing

The capability of adding materials layer by layer enables the user to fabricate complex patterned objects in a short amount of time[36]. It also allows manufacturing of objects in small amount which was not economically feasible previously. Other advantages include customised parts, less tooling equipment and minimum technical expertise needed to operate 3D machines[37]. The building time varies with each 3D printing machines or technique. Various factors such as material used and print settings will affect the final resolution of the build part. High equipment cost, limited printable materials, pre- and post-processing requirements are some of the disadvantages linked with 3D printing. However, new methods and materials are currently being developed by researchers[38].

2.1.3 Types of 3D printing mainly used for biology

Even though most 3D printing technologies (Figure 7) are well-established and commercially available[35], its use in the biology field is just being explored. Indirect printing technologies are known for its precise control over both external and internal macro/microstructure but it does not allow printing of cells directly. It is mainly used for fabricating drug delivery systems, potential biochips or biosensors. However, each technology has its own advantages and disadvantages. Technologies are grouped according to the way materials are joined together. Light-based systems such as stereolithography (SLA) and polyjet inkjet-based systems use ultraviolet (UV) for curing photopolymer. While laser-based system involves high power lasers for sintering or melting (Selective Laser Sintering, and Selective Laser Melting). Binding-based system such as 3DP uses glue to join the material. Due to its ultrashort pulses, two photon polymerisation (2PP) or near-infrared system are able to fabricate microstructures such as the “lockyballs” interlock scaffold[39]. Many papers highlighted the extensive use of indirect 3D printing in the fabrication of scaffolds/implants[35]. Although complex and high precision geometry can be achieved, cells are still seeded secondary, resulting in the inability to replicate a multi-cellular structure[40].

Figure 7 3D printing technologies based on the classification mentioned and method to form the structures. Figure taken from [35]

This leads to the creation of a new subset known as direct printing. Direct printing or Bioprinting has been gaining huge interest in the field of 3D printing as cells can be patterned or printed with precise control and built into 3D tissue architecture in the microscale[35]. For this chapter, they are classified according to the way in which biomaterial is dispensed; (i) Inkjet, (ii) Extrusion and (iii) Laser Assisted Based (LAB). Many commercial printers that are currently available such as NovoGen MMX Bioprinter™ and regenhu BioFactory® carry multiple print heads together (inkjet or extrusion) allowing multiple cell types and biomaterials to be printed directly in a specific 3D arrangement. Any LAB technique consists of three parts: a pulsed laser source, a target plate (Quartz ribbon) and a biopolymer hydrogel or cell suspension. There are two widely active variation: Matrix-assisted pulsed laser evaporation direct write (MAPLE-DW) and Biological laser printing (BioLP) which are distinguished by the nature of the ribbon[41].

Table 1 describes some methods of 3D printing technologies with the materials currently used in biology while comparing the advantages (Adv) and Disadvantages (Dis) and resolution of the parts. The applications column shows the structures/devices that were made without additional tooling.

Table 1 The different types of 3D printing technologies, their energy source (energy required to join the material), materials that are compatible with the machines, advantages and disadvantages of the machines, and applications in the fields of biology.

3D printing	Energy Source	Cell Printing	Materials	Resolution (μm)	Adv	Dis	Applications
SLA	Laser/UV	Yes	Photocurable resin/polymer-Acrylonitrile Butadiene Styrene (ABS) like, etc.	0.5 to 50	High accuracy with complex internal features	Single material; requires photopolymers	Hearing aid shells[42], Hemi knee joint[43], patient skull for casting,[44] tooth/denture[45, 46] Making of master mould[47]Microfluidic chips with active features[48, 49]Microfluidics Interface (MFI)[50]Pathogen detection[51, 52] Biological assay (cell observations)6[53]
DLP	UV	Yes	Photocurable resin/polymer	0.5 to 50	High accuracy with complex internal features	Single material; requires photopolymers	Making of master mould[54] Cancer assay (studies on cell migration)[55]
2pp	Femtosecond Laser	Yes	Photocurable resin/polymer	up to 100nm	High accuracy with complex internal features	Single material; requires photopolymers	Lockyballs[39] Ossicular (bone in the middle ear) prosthetics[56]Biological observation on cell mobility[57, 58]

Inkjet	UV	Yes	Photocurable resin/polymer	20 to 100	Low cost; fast build rate, multiple materials	Viscous solution may clog system	Making of master mould[59]Versatile chips for different types of electrodes for gas detection[60]Toxicity assay[61]Biological assay (cell observations)[53]
Bioprinting	Laser/UV	Yes	Photocurable resin/polymer	20 to 300	Multiple materials, cells can be printed as well	Low build rate, extrudes as filament only, viscous solution may clog system	Soft scaffold patterning of C2C12, PC12, Smooth muscles cells, Human fibroblast and bovine aortic endothelial cells,[40, 62]Cartilage[63] Making of vascular channels [64-67] Soft scaffold patterning of human fibroblasts and bovine aortic endothelial cells Liver construct[40],[62] heart or vessels constructs[68-70] Soft scaffold patterning of rat bladder cells or cell microarrays[36]

FDM	Thermal	No	Thermoplastics such as ABS, polycarbonate, and polyphenylsulfone;	200 to 500	Cheap materials, ease of support removal	Slow build time, restricted accuracy. Not many transparent materials available	Pathogen detection of bacteria[71]and viruses[72]
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2.2 APPLICATIONS OF DIFFERENT 3D PRINTING TECHNOLOGIES

The environment where cells subside is very important. It contains many different physical [73] and chemicals signals[74] that either determine cell fate or behaviour. With 3D printing, not only a 3D environment can be created for the study of cell interactions, patterning or spatial space cells in specific location can also be achieved. This allows researchers to have a better understanding of cell behaviour. In the following section, some of the potential 3D printers that can aid in creating a controlled environment for cells studies will be discussed. These machines were chosen either due to their ability to fabricate high resolution structures or they are have the potential to print cell directly in the near future.

2.2.1 Bioprinting

3D bioprinters are able to customise 3D scaffolds for tissue engineering, it can pattern biological materials including DNA, proteins or cells either directly or on the scaffolds[35]. Currently, commercial bioprinters such as regenHU BioFactory®, EnvisionTEC 3D-Bioplotter® or NovoGen MMX Bioprinter™, combines multiple print heads, enabling multiple cellular structures to be made. The print heads can be grouped as dispensing or jetting[75]. For dispensing, air pressure or physical pressure is used to force the material through a nozzle in a controlled environment, creating a filamentous form. Solidification of the deposited materials for mechanical integrity is done through physical or chemical crosslinking. Depending of the machine set up, either the print head or the stage is moving while the material is dispensing[75]. The biomaterials are usually viscous hydrogels encapsulated with the cells for maintaining their shape before crosslinking. Jetting is defined as creating droplets of the materials for higher resolution. The technology commonly used are inkjet or microvalve. Inkjet dispense biomaterials in droplet form while the droplet size can be controlled either by thermal or piezoelectric means[75]. For microvalve printing, simple droplets or filamentous forms can be created using pneumatic pressure. A small valve controlled mechanically, electrically or magnetically open or close allowing the flow of material through the nozzle[75].

Biological implants consist of mainly cellular materials such as cells, ECM matrix protein and hydrogel. These implants also known as organ printing have huge potential in tissue engineering as autologous cells are being used thus reducing the possibility of rejection by the body and waiting time for obtaining a suitable organ. Organ printing is a computer-aided process where cell-laden biomaterials or cell aggregates serve as building blocks and are

assembled into a 3D functional organ through the bioprinter[70]. By placing the multiple cell types at specific location to mimic the patient's organs, possible patient's organ can be manufactured commercially in the near future. However, more research have to be done still specifically in three areas (i) better understanding of specific cell functionality for example, how cell interacts with the material or how the printing process may affect cell behaviour (ii) Combinations of different biofabrication techniques for production of tissues and organs since various biomaterials available may not be suitable for printing (iii) performing characterisation studies on immunology, toxicity and organ integrity after printing [70]. Although organ printing shows huge potential as mentioned, the three areas need to be addressed. Currently, there is no fully functional biological implant reported. However, advancements have been made in the field of organ printing, heart vessels (Figure 8) and tissues[68, 69], cartilage[63], skin[76] and liver tissue[40] are some of the organs in the works.

Figure 8 Bioprinting of blood vessels. A) Bioprinter loaded with cells and support B) Printing and patterning of support (purple) and cell (orange) in filament form C) formation of blood vessels. Picture adapted from [68].

One possible way for carrying out characterisation is the use of organ-on-chip. The chip can provide a conducive microenvironment that is similar to the human organ to simulate how cell might behave in any given conditions[77]. There are both single organs-on-chips and multi-organs-on-chips available. Multiple single organs-on-chips can be integrated together to imitate and simulate biological system required by the user[75]. To fabricate organs-on-chips, cell culture techniques were combined with soft lithography before the rise of Bioprinting[62]. The advantage of the bioprinter over conventional fabrication method is the ability to print the chip with one machine, saving time and money[75]. At the same time, the limited geometries available with PDMS chips, have made Bioprinting a better option[78, 79]. Currently,

researchers are focusing on creating vessels by using sacrificial materials and removing them physically by mechanical pulling or vacuum, or by chemical means once the vessels is made[75]. Some sacrificial materials include fugitive organic ink[65, 66, 80], gelatin[67] and carbohydrate glass,[81] with different bioprinters. Recently, a fully functional hydrogel microchannel was attained using the NovoGen MMX bioprinter[64]. GelMA hydrogel microchannel which was bioprinted can possibly replace blood vessel. Bio-compatibility of the channel and cell viability was imaged using a fluorescence microscope at the end of each day. It was observed that cells were proliferating better inside the channel than in ordinary hydrogel blocks (Figure 9), providing researchers with another fabrication method for in vivo environment studies for cell survival, division and differentiation.

Figure 9 (Left) Schematic representation of bioprinting of agarose template fibres and subsequent formation of microchannels via template micromolding. A) A bioprinter equipped with a piston fitted inside a glass capillary aspirates the agarose. After gelation at 4 °C, agarose fibres are bioprinted at predefined locations. B) A hydrogel precursor is cast over the bioprinted mould and photocrosslinked. C) The template is removed from the surrounding photocrosslinked gel. D) Fully perfusable microchannels are formed. (Right) Photographs of the different bioprinted patterns. A-i) planar bifurcating pattern, B-i) 3D branching pattern and C-i) 3D lattice pattern. Green refers to the bioprinted template with agarose while red refers to microchannels perfused with a fluorescent microbead. Picture taken from [64]

2.2.2 Stereolithography (UV based printing)

The first commercialised stereolithography 3D printing machine was made in 1988 by Chuck Hull[82]. It is a layer by layer UV laser based (wavelength around 350nm to 400nm) system which scans and traces over a certain area to cure the photopolymer resin material. The material absorbed the energy from the lasers and polymerisation occurs to form the desired pattern. Building the next layer requires the stage to move in the z-direction according to the thickness of the layer. Once the stage is set, a sweeping blade recoat the material and the laser starts to trace the pattern of the next layer. This recurring process continues until the 3D part is completed. SLA fabrication is known for its high precision and surface resolution. Nonlinear effects due to high powered laser should be controlled. With the recent expiring of patents involving the SLA systems, it has become a highly commercialised and popular 3D printing machine in the world[30, 83, 84]. Improvements made to SLA systems lead to fabrication of high-resolution products, reducing production cost as well as material usage[30, 83, 84]. For example, the personal Form1+ type high-resolution 3D printer is affordable and with constant improvements in its resolution and accuracy, it is able to achieve industrial precision standards, with feature size as small as 300 μ m[30]. One huge disadvantage of SLA systems is the use of short wavelength for polymerisation which is toxic to cells. However, its fast speed and high accuracy allows easy and efficient fabrication of microfluidic devices or scaffolds before cell seeding.

An innovative 3D immunomagnetic flow assay on-a-chip was designed and fabricated by Lee and team using the SLA system[51]. This study is an outstanding example of how 3D printing combined with microfluidics can aid in the clinical field and not just being a prototype[75]. A cylindrical 3D microchannel referred to as High-capacity Efficient Magnetic O-shaped Separator (HEMOS) was printed in the laboratory without a need for a clean room. The trapezoid cylinder reduces linear flow velocity, allowing the handling of up to 150 patients samples within 1 hour. The addition of Antibody-Immobilised Magnetic Nanoparticle Clusters (AbMNCs) to the clinical samples will form an AbMNCs–Bacteria complex with Salmonella. A high magnetic force can then be applied to the walls of the HEMOS attracting the AbMNCs–Salmonella complexes, thus capturing the bacteria on the sides of the HEMOS shown in Figure 10. Simulations on the influence of the magnetic arrangement in the HEMOS was also carried out in the study. Recently, detection of *E. coli* in milk was done using the same device with slight modifications, the finding was published by Lee and team as well[52].

Figure 10 (a) Schematic illustration of separation of captured bacteria by inertial focusing. (b) Illustration of Dean vortices in a channel with trapezoid cross-section. (c) Photograph of the 3D printed device. Picture taken from [52]. (d) Schematic illustrations of 3D immunomagnetic flow assay. The magnet–spacer assembly was placed in the opening of the HEMOS. Picture taken from [51].

Another system that is like SLA is Digital Light processing system. It combines DMD-PP technology which is capable of printing materials by creating dynamic mask for each layer [85]. Instead of using a laser source, an UV lamp or LED is used (405nm or 385nm). A PEGDA hydrogel was printed like honeycomb structures to mimic the 3D vascular morphology of an in vivo microenvironment. Metastatic properties of HeLa cancer cells were examined in the honeycomb structures. It was observed that the migration speed of cancer cells decreases as the width of the microchannel increases. The authors concluded from the experiment that cancer cells may be travelling faster in smaller veins than in large arteries. This showed that with 3D printing, complex bio-environment can be realised[75].

2.2.3 Direct laser writing/Two photon polymerisation (NIR based printing)

Polymerisation is defined as a process where a large volume of small molecules is joined together to form a big molecule. The process of two-photon polymerisation (2PP) or direct laser writing (DLW) is like the SLA process. A laser is used to excite photoinitiator molecules, creating free radicals which causes chemical reactions between photoinitiator molecules and monomers/oligomers within a transparent resin. The key difference between the SLA and the 2PP process is the use of two-photon absorption for excitation of photoinitiator molecules shown in Figure 11. To achieve two-photon adsorption, a femtosecond laser is utilised[86, 87]. A virtual state is created where two-photon of same or different frequency are absorbed by the photoinitiator (Figure 11). This enables longer wavelength (near-infrared range) to possess a similar electronic excitation like a single photon which requires much higher energy[86, 87]. This nonlinearity of two-photon absorption not only excites the photoinitiator molecules with less energy but material crosslinking can occur within the immediate vicinity of the focal volume[88], leading to significantly higher resolutions than other direct write techniques (up to 100 nm)[88].

Figure 11(a) Mechanism of TPA when simultaneous excitation. (b) and (c) Illustration of two methods for increasing the probability that TPA occurs: density of photon is increased by (b). spatial compression using objectives with high numerical aperture, (c). temporal compression using ultrafast lasers. Picture taken from [89]

Two-photon polymerisation provides several advantages over conventional processes especially in the fabrication of small medical devices. There is a wide range of near infrared transparent materials such as GelMA and PEG-DA which are biocompatible and suitable for tissue engineering[90]. The cost should be reduced as no cleanroom facilities and specialised equipment besides the laser is required [56] as compared to other fabrication techniques such as deep reactive ion etching and lithography[91]. This flexibility may lead to fabrication of a medical device within proximity of an operating room or another clinical site. However, due to the tracing method, the speed is slower as compared to DLP. Despite 2PP being a relatively new technology, it is expanding into the field of micromechanical systems, microfluidic devices, biomedical devices and scaffolds for tissue engineering.[35, 75].

Besides its usage as a diagnostic device for the detection of bacteria, microfluidic devices can also aid in the observations of microorganism mobility. Some bacteria contain a flagellum which acts as a sensor, aiding the bacteria in moving towards or away from the signal of interest. A 3D microfluidics device known as a nanoaquarium was printed by Midorikawa and his team with DLW for learning of mobility in microorganisms, *Euglena*[58] and *Phormidium*[57]. By combining DLW with dry and wet etching, microchannels and micromirrors were produced in glass chips. This allowed them to focus easily on the bacteria with the microscope and reduced the amount of reagent required. Minute quantities of dissolved chemical substances can also be further analysed. DLW allows rapid prototyping of various nanoaquariums with different features, resulting in better observations and dynamics mobility of microorganisms analysis[58].

Bacteria live in the nature as small, densely packed aggregates containing $\sim 10^1$ – 10^5 cells[92]. Aggregates in larger bacterial communities display non-random spatial organisation, and recent evidence indicate that this spatial organisation is critical not only for fitness but resistance to antibiotics and serve as building blocks for larger biofilm communities[92]. The advantages of direct printing bacteria rather than using the “lobster traps[93]” is the ability to organise complex communities of cells composed of physically segregated bacteria, still chemically communicating with different populations[92]. By using DLW with gelatin, it allows defined polymicrobial communities to be constructed in a limitless variety of 3D geometrical arrangements and enable sociomicrobiological interactions within these communities to be studied at an unprecedented level of detail[92].

Spatially controlled 3D microstructures containing protein binding ormocomp cubes and protein repellent PEG-DA scaffold has been fabricated by DLW. As predicted upon the cultivation of fibronectin, ECM protein preferentially binds to the ormocomp part, followed by the culture of fibroblasts on the scaffolds. Interestingly Klein *et al.* have found that cells were adhered to Ormocomp or connected through the several cubes and studied via confocal microscopy (Figure 12). 3D growth pattern of cells within the interconnected can be observed. Immunostaining by actin has demonstrated the periphery of the adhered cells and the ormocomp cubes not on PEGDA part was revealed by paxillin staining. It was demonstrated that the cells cultured in these scaffolds specifically form cell adhesion sites in the ECM functionalised sections[94]. This research suggests future possibilities of systematic studies of spatial ligand distributions and mechanical scaffold stiffness on cell behaviour in 3D environments.

Figure 12 Scanning electron microscopy images illustrating the fabrication of 3D composite polymer scaffolds by sequential DLW. a) In the first step, 3D frameworks consisting of protein-repellent PEG-DA with 4.8% PETTA are polymerised and developed. b) Frameworks are then cast with the photoresist Ormocomp and cubes (one cube is highlighted in red) are precisely attached to the PEG-DA beams in a second DLW step. c) Higher magnification image of an Ormocomp cube (highlighted in red). Picture taken from [94].

Cell-matrix invasion is an important part of the pathological process which includes leukocyte extravasation and cancer cell metastasis. In recent years, cell matrix invasion was studied by the migration of cells within the polymer scaffolds which plays the role of ECM. In order to study the cell matrix invasion, Greiner *et al.* have fabricated the well-designed polymer cell scaffolds DLW as shown in Figure 13[95]. It is a well-known fact that cells change their morphology while migrating through the pores of ECM during embryogenesis, wound healing, or metastasis. Greiner *et al.* have studied the effect of nucleus stiffness on scaffold invasion. Thus, 3D printed scaffolds

with predefined pore sizes can be used for cell migration and invasion studies with different ECM molecules. Moreover, 3D printed scaffolds can be potentially used for screening and determining cancer stages based on the nuclear mechanics and matrix invasion.

Figure 13 Microporous 3D cell culture scaffolds produced by direct laser writing (DLW). Scanning electron microscopy (SEM) images of 3D PETTA scaffolds with mesh sizes of 2 mm, 5 mm, and 10 mm fabricated by DLW. Picture taken from [95]

2.3 CURRENT ISSUES AND FUTURE APPLICATIONS

Advancement in 3D printing hardware, biomaterials and computer software have led to an uptake of 3D printing in life sciences, creating new possibilities in which scientific research can be conducted[35]. 3D printing will require collaborations and integration of different disciplinary approaches while converging the multiple skills sets in order to take it to the next level[36]. Currently, the uptake of 3D printing in the field of biology is on the rise. Researchers are coming up with systematic approaches to determine biocompatibility and printability of biomaterials. Settings on the printers will also require optimising. In this section, some of the advancement in biomaterials, hardware, software and post processing which will aid 3D printing to be a killer application in biology will be discussed.

2.3.1 Biomaterials

The print ink or in this case, biomaterials, is an important component in 3D printing. While new materials are appearing every day on the market, materials compatibility with either biologicals or printers still remain an issue. Research is being done to either discover new materials or improve on existing materials[35]. To address these issue, Bens and team developed a new photopolymer resins for the SLA system. By adjusting the formulation, the polymer can be both soft or hard plastics[96]. Its low-viscosity, high accuracy speeds coupled with low toxicity make it better against other acrylic resin materials such as PP, PE or ABS[96]. This saves cost as it only needs one material to obtain different mechanical properties. Another method to improve the 3D printed material is the additional of nanofillers such as graphene. Graphene is an atom-scale carbon sheet arranged in a honeycomb lattice which exhibits a unique combination properties in term of high chemical, electrical, thermal conductivity and stability, improvement in stiffness resisting tear and abrasion, gas permeability, protection against Ultraviolet (UV) and Infrared (IR) Radiation[97-100]. Recently, Tolle and team were able to print graphene with an inkjet printer or bioplotter (extrusion based system)[101], providing the opportunity for fabrication of scaffold with multiple functional properties. PDMS was first used for academic laboratories in the late 1990s. Its high gas permeability, transparent optical properties, reasonable cost, rapid fabrication and bio-inertness [102] make it a popular material among glass and silicon in the field of biological microfluidics[103]. However, PDMS is not printable at the moment still. As mentioned, SLA and 2PP require a photopolymer resin that alters its physical and chemical properties when exposed to light[103]. In addition, material tuning abilities and range of transparent material propel it to be preferred choice for 3D printing microfluidics. One example was done by Paydar and team for a multifunctional microfluidics device from the commercial Objet printer. They reported being one of the first in making a microfluidic interconnect by printing a flexible elastomer O-ring made from TangoBlack® and a rigid plastic body from VeroBlack® for mechanical clamping onto the chip. The key advantage will be cost efficient and time saving as the entire device composing of both clamp and gasket can be fabricated at one go without the need of additional manual assembly[104]. However, most materials for 3D printing are proprietary, require additional optimisation and characterisation before any biological experimentations. Feng Zhu and team investigated some commercial materials biocompatibility from the multi-jet modelling (MJM) system and the SLA systems. For MJM, VisiJet Crystal (rated United States Pharmacopoeia (USP) Class VI) while SLA the clear photopolymer, Watershed 11122 XC (Watershed) and Dreve Fototec 7150

Clear (Drewe Otoplastik GmbH) were used for experimentation. For testing biocompatibility of the materials, microfluidic devices with different material were printed and seeded with Zebrafish embryo. Two materials VisiJet Crystal (HD3500+) and DSM Watershed (Viper Pro) were found to be toxic to the Zebrafish embryos when grown for more than three days and even after post treatment with ethanol. However, Drewe Fototec material which was initially toxic to zebrafish embryos, was found to be completely inert towards zebrafish embryos when soaked in 99% ethanol for 24hours[53] after printing. This showed that commercial 3D printed materials must be properly treated before using with biologics. With further tuning and understanding of materials science, multi-functional materials can be compatible with existing 3D printing technologies.

2.3.2 Hardware

As shown in the earlier section, one key limitation of 3D printing is that most commercial printers can only print complete 3D part with one material. This may reduce the functionality of the structures, affecting the uptake rate of 3D printing. With constant improvement in the hardware, certain 3D printers such as the bioprinters are able to print multi-materials for a single part. With this advancement, manufacturing time and cost can be reduced and new products can be developed. Lately, Mannoor and team were able to intertwine biological cells with functional electronics flawlessly using the 3D printer[105]. This may lead to the creation of innovative bioimplants in both form and function in the field of biosensors and drug delivery system. Future bionic implants can be expanded with 3D printing by patterning or integrating nanoparticles and cells seamlessly[105]. Due to the layer by layer process, “pyramid steps” may be seen on the microfluidics chips affecting its functionality. Au and team did a comparative study between soft lithography and SLA systems for the fabrication of microfluidics devices comprising of integrated female Luer connectors and different microchannel sizes[106]. SLA made devices are quicker, cheaper and complex geometry can be done unlike soft lithography. The limited laser spot size of 100µm prevented microscale features to be produced and the resolution was reasonable. One way to improve the resolution and speed of the 3D printing system will be to combine it with other 3D printing machinery. 2PP can create very fine structures due to its ultra-short pulse laser but its tracing speed is slow. Therefore, by combining a conventional laser for manufacturing the device with 2PP will allow it to create very fine features. Hengsbach and team fabricated microfluidics device for studies on cell motility with different micro-textured surfaces[107]. Hence, combining with other

technologies, better device can be built with quicker speed. Depending on the 3D printing system, machine price ranges from \$2000–\$10 000. Besides the high cost of the printers, proprietary materials also prevent the user from greater flexibility for experimentations[84]. Shallan and team were able to fabricate a fully functional microfluidic device with a micromixer, gradient generator and droplet extractor for isotachopheresis using a low-cost consumer 3D printer[49]. This showed with more research, low-cost consumer printer would be able to produce good quality chips. Another solution will be the use of discrete elements that enables designers to build large-scale microfluidic systems in 3D. Bhargava and team created a sample library of standardised components and connectors that can be manufactured using stereolithography and assembled into a chip. This allows chips to be modular, diverse, and predictable by simple network analysis techniques[108]

2.3.3 CAD software

Reverse engineering (RE) compliments 3D printing very well as it allows the user to obtain exact model or modify the existing models. By combining RE technology for the scanning of patient's body parts and 3D printing of the scanned parts, human body tissue or organs similar to the patient can be obtained. For example, bone structures can be captured by a 3D scanner, CT scanner or MRI system and be converted into a 3D model. Commercial programmes on the market such as Mimics (Materialise NTV) is available for generating 3D model from MRI or CT scanned images. It will be easier for researchers to do further modification or redesigning on the 3D scanned models using Rhino, Solidworks and Medical modeler etc. rather than drawing the whole design themselves. More details about the designing and simulation process can be found in [35]. Most software requires users to design on a 2D plane for 3D structures which can be a challenge especially for biologists. A survey conducted by Bourell in 2009 showed that the uptake rate for 3D printing may be reduced due to the lack of competent CAD software available to researchers, thus hindering their research in the long run[38]. To entice higher acceptance for 3D printing, CAD interfaces have to be improved to make it more user friendly by having pre-defined tools[38]. To fully exploit the advantages of 3D printing, one way is to integrate process–structure property relationship into CAD systems. Computational tools such as multi-scale modelling, inverse design and optimisation methods will be necessary for simulating and analysing materials properties. New CAD systems need to be simplified significantly for easy decision making and understanding for the user. With constant research in the software department, 3D printed parts can achieved significant improvement in terms of

performance that fully utilises materials efficiently, reducing production cost and possibly unrealisable capabilities[38].

2.3.4 Automation of pre-and post-processes

The automation of pre/post processing could simplify the improvement for 3D printing. Cells based device and implants are subjected to contamination from the outside environment due to manual intervention and transferring. The whole process can be automated and be done in a clean room. For example, machines or robots can be used for the different processes of organ printing. After cultivating the cells, it could be directly transferred into the bioprinter. 3D hydrogels tissue construct can be placed directly into a bioreactor by a precision robotic arm for cell growth. With the integration of all the different phases within a same area or machine, contamination can be minimised. In the future, printing of organs may also follow the production line concept. Doctors with access to the patient's anatomy images can create implant immediately with a click of a button. Images are first converted into a 3D CAD file and sent directly to the 3D printer. Once the part is done, he or she can check on implant immediately from the cleanroom. The cost and time saved with 3D printing will be a significant development in the medical field.

2.4 Final comments

Although 3D printing has made huge advancement in biological field, more research need to be done on materials and printing techniques for it to be usable. As mentioned, most 3D printing machines can only print one material at a time, it is time consuming to change the material. There are many materials such as plastics and metals that are currently available on the market, however many of these materials are not tested with 3D printing. Each material may require a different printing technique to get the same properties as traditional techniques due to the fabrication of structures layer by layer rather than bulk subtraction. In the next three chapters, detailed elaboration on the systematic approach in researching ways in terms of materials and printing technique, to create the environment for cell-cell interactions studies will be touched on. Both mammalian and bacteria cells were used.

CHAPTER 3: 3D PRINTED SCAFFOLD FOR CARDIAC TISSUE ENGINEERING

3.1 INTRODUCTION

Tissue Engineering is defined as an interdisciplinary field which involves the use of cells and a scaffold/matrix to develop new functional tissue for implantation back to the donor[109, 110]. Three-dimensional (3D) scaffolds play vital roles in tissue engineering such as drug delivery systems, investigation of interaction between cell behaviour and material[111] but also essentially provide a biological environment for cells to readily attach, proliferate and maintain their differentiated phenotype and allow deposits of new tissue matrix throughout the entire construct[112, 113].

Polymeric biomaterials such as polyurethane (PU), polylactic acid (PLA), polycaprolactone (PCL) and poly(L-lactide-co- ϵ -caprolactone) (PLC) are commonly used in tissue engineering for their unique mechanical, biocompatible, biodegradable and nontoxic properties[114-119]. In recent years, PCL based biomaterials have been extensively used in biomedical, pharmaceutical, controlled drug delivery and tissue engineering applications[120-124]. Although PCL is well-known for its biocompatibility, biodegradability, mechanical and structural stability, lack of bioactivity and surface energy (high hydrophobicity) have led to reduced cell affinity and small tissue regeneration rates[125]. One of the strategies to improve PCL is to incorporate nanocomposites of nanoclay[126], graphene[127] or CNT[128-130].

The incorporation of reinforcing agent such as nanoparticles in the polymer matrix further stimulates the thermo-mechanical and biological response[131-133]. Multi-walled CNT (MWCNT) possesses exceptional mechanical, electrical and optical properties. Its high aspect ratio structure makes it useful as a nanofiller in the fabrication of polymeric nanocomposites, possibly leading to improved mechanical properties, biocompatibility and reduced cytotoxic effect for biological applications[134]. The regenerative capabilities of the adult human myocardium when compared to the blood or skin are significantly minimal and insufficient to compensate for the loss of cardiac myocytes. A right combination of cells, matrix, molecular and physical regulatory factors have to be present in order for inducing tissue development and remodelling of the myocytes[135, 136].

PCL nanocomposites (nanoclays, graphene, CNTs) are prepared using methods such as solvent casting, freeze drying and electrospinning[127, 129, 130, 137-142]. However, these existing fabrication methods are unable to produce scaffolds of desired control pore sizes. Recently, research groups have started looking into the use of 3D printing in the field of biology[75, 143-145]. In 3D printing, models are designed on CAD software or obtained through a CT/MRI scan which is then converted to a Stereolithography (STL) file. The STL file consists of a series of 2D cross-sectional layers, creating a path for the printer to take for tracing[143]. This layer-by-layer approach can generate spatially-controlled cell patterns, fabricates complex geometry scaffold and printing of multi-materials. These advantages give 3D printing its newly acquired popularity[146-149].

Printing of PCL and CNT scaffolds in conjunction with the 3D Bioprinter will be discussed in this chapter. PCL and its composites scaffold with well-defined structure have been successfully designed and fabricated using a 3D printer. The improved thermal and mechanical properties of printed scaffolds with various percentages of CNTs have been reported. The biodegradation behaviour of PCL and its CNT composites have been examined in enzymatic media. Furthermore, cytotoxicity and cell compatibility of PCL-CNT composites have also been studied. Initial results showed that the 3D printed PCL and its CNT composites have the potential to be used in cardiac tissue engineering.

3.2 MATERIALS AND METHODS

3.2.1 Materials

PCL ($M_n = 80000$) was purchased from Sigma-Aldrich. Functionalised MWCNTs (20-30 nm outer diameter, 5-10nm inner diameter with 10 to 30 μ m length) was purchased from Chengdu Organic Chemicals Co., China. Chloroform, [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] (MTT) and other chemicals were purchased from Sigma-Aldrich.

Cardiac H9c2 cells of rat origin were derived from the same initial CRL-1446 cell culture obtained from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (SH30243.02, HyClone™, GE Healthcare) supplemented with 10% fetal bovine serum (F1051 Sigma-Aldrich), 1% Penicillin-Streptomycin (P4333 Sigma-Aldrich) and grown at 37°C in a 5% CO₂ humid atmosphere.

3.2.2 Nanocomposite Preparation for printing

The nanocomposites were prepared by sonication by adding well dispersed CNT solution and PCL in chloroform. Different amounts (1, 3 and 5%) of CNTs were determined according to w/w% of PCL and sonicated (*Model 505 Sonic Dismembrator from Fischer Scientific*) with predetermined volume of chloroform for 10 min. The well-dispersed CNT solution in chloroform was mixed in PCL with chloroform solution and sonicated for 30 min at maximum power. The PCL/chloroform ratio was kept constant to 8% (w/v). Pure PCL (8% w/v) was also dissolved in chloroform and sonicated for 30 min. Hereafter, nanocomposites will be termed as PCL-CNT-1, PCL-CNT-3, PCL-CNT-5 using 1, 3, 5 % CNT as the nanofiller, respectively.

3.2.3 3D printing of PCL and PCL-CNT scaffolds with RegenHU Bioprinter

PCL and its nanocomposites were fabricated and printed by RegenHU Bioprinter Biofactory Machine (Switzerland). A grid structure was designed using BioCad software. The structure covering 20 mm × 20 mm consisted of two horizontal and two vertical lines separated by 1 mm.

Once PCL and its nanocomposites solution have been sonicated, solution were immediately charged into two 5 ml syringes, which were then connected to a gauge 21 nozzle tip and the dispenser. The substrate for printing the biomaterial can be fixed on the stage by turning on the vacuum selection. The pressure of the 3D printer was first set to 1 bar for extrusion of the PCL and was tuned for the systematic free flow of loaded material (nanocomposite) accordingly. The syringes containing the 8% PCL solution or with its nanocomposites were connected to the nozzle and calibration with the NLM testing (distance between the needle and the glass slide) was done before dispensing any solution out of the syringe. For this bioprinting system, the feed rate refers to the speed in which the stage moves (x, y- direction) allowing for the material to be deposited while the thickness refers to the stage moving in the z-direction after each layer is printed. As the feed rate of the 3D printer is an important parameter for printing well-defined patterns, feed rate values of 800, 1000 and 1200 were used while keeping the other variables constant. Several batches of PCL and its nanocomposites were printed using an optimised feed rate. The PCL and its nanocomposite grid scaffolds were dried under vacuum for 24h before performing any characterisation or testing. Microscopic images of the scaffolds

were captured using Stereo Microscopes with a 4X magnification (*Olympus SZX7 and Zeiss AxioTech*).

3.2.4 Raman Spectroscopy

Raman spectroscopic measurements were carried out in backscattering geometry using an Ar⁺ excitation source of 514 nm wavelength coupled with a *Renishaw inVia Raman microscope*.

3.2.5 Thermal Characterisation

Differential scanning calorimeter (DSC) *TA-DSC-Q-200* was used to measure the melting and crystallisation temperatures along with the heat of fusion and crystallisation of the scaffold materials. All the samples were heated and cooled at a scan rate of 10°C/min in the first run to obtain the peak temperatures and enthalpies. A repeat second heating run was carried out.

3.2.6 Mechanical characterisation

Nanoscale mechanical properties of PCL, PCL-CNT-1, PCL-CNT-3 and PCL-CNT-5 nanocomposites were studied using a nanoindenter (*NanoTest Micromaterials Ltd, Wrexham UK*) with a 150 nm Berkovich pyramidal tip. The elastic modulus (*E*), maximum load and hardness were obtained. Each sample was indented more than 5 times at different regions separated by a few millimetres.

3.2.7. Electrical Conductivity

Conductivity of PCL-CNT nanocomposites was studied by using Loresta-GP MCP-T600 from Mitsubishi Chemical Analytech.

3.2.8 Enzyme Degradation

The enzymatic degradation of PCL and its CNT nanocomposite scaffolds was performed at 37°C in potassium phosphate buffer solution (PBS) (pH 7.4) by *Pseudomonas Lipase (type XIII)*. Scaffolds having dimensions of 5 × 5 × 0.15 mm³ were placed in a small tube containing 0.1 mg/mL *Pseudomonas lipase* with 3 mL of PBS. Enzymatic reaction was carried out for 3, 6 and 24 h after incubating the tubes at 37 °C with continuous shaking. The scaffolds were then

washed with phosphate buffer and dried in vacuum oven. SEM images before and after enzymatic degradation were captured by using *JEOL JEM-5600LV* scanning electron microscope.

3.2.8. Biological compatibility

Cell viability test with the scaffold materials was done using the Vybrant® MTT Cell Proliferation Assay Kit by Thermofisher with a few modifications. Briefly, 50,000 cells were seeded onto the scaffold and placed into a 24-well plate with 1ml of medium. After four days of cell seeding, the spent media was removed and 400µl fresh medium and 40µl MTT solution (component A) were added to each well. After 4-hour incubation at 37°C in 5% CO₂, MTT solution from each well was carefully removed. Instead of using Component B from the kit, 250µl DMSO (Dimethyl Sulfoxide) was added to each well. The absorbance of solution was then measured at 540 nm using the TECAN Infinite 200 PRO plate reader.

To evaluate cell viability with the pure PCL and PCL-CNT nanocomposite, the live/dead viability/cytotoxicity kit, for mammalian cells (Cat no L3224) from ThermoFisher scientific was used. H9c2 cells (~50,000) were grown together with PCL and PCL nanocomposite in DMEM media for 4 days in a 24 wells plate. After 4 days, the wells were washed twice with PBS followed by the addition of 200µL of 2 µM Calcein AM and 4 µM EthD-1 solutions from the kit in the wells and incubated for 30 min at 37°C. *Zeiss Axio* fluorescence microscope was used to observe the cells inside the wells.

3.2.10 Cell imaging on Scaffold

Cells seeded on the PCL and PCL nanocomposite scaffolds were grown in DMEM media for 4 days. The scaffolds were washed two times with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. After washing off the paraformaldehyde, 0.1% triton X was added for 5 min. Scaffolds were then incubated for 30 min at room temperature with ActinGreen™ 488 ReadyProbes® Reagent and NucBlue® Fixed Cell ReadyProbes® Reagent from Thermofisher Scientific for staining F-actin and the cell nucleus respectively. After three washes with PBS, the scaffolds were transferred onto a glass slide where the images were captured by a Zeiss Axio fluorescence microscope.

3.2.11 Statistical analysis

Data are presented as the mean $\pm$ standard deviation compared using one-way ANOVA Dunn's multiple comparison test. All data were analysed using Prism software (version 6.01). $P < 0.05$ was considered to indicate a statistically significant difference.

3.3 RESULTS AND DISCUSSION

3.3.1 3D printing of scaffolds

Images of 3D printed scaffolds of PCL and its nanocomposites are shown in Figure 14a. Conditions for 3D printing have been achieved by changing the pressure, speed (feed rate) and needle size. Optimised conditions for different feed rates with constant temperature, pressure and needle size have been compiled in Supplementary Table 1. Printed PCL and its CNT composite scaffolds with different feed rates have been compiled in Supplementary Figure 1. To determine the optimisation, two factors were considered: the line width during printing as well as the square that is created from overlapping vertical and horizontal lines. Well-defined structures for each composite is determined when overlapping line width does not smudge and the square are constant through of the scaffold. The default feed rate and thickness were optimised at 1000 and 0.20 μm respectively for our further studies. The grid structure of the PCL and its nanocomposites becomes more prominent with increasing CNT percentage. The nanotube and PCL matrix interaction plays a vital key role; CNT acts as a nanofiller and holds the PCL matrix to obtain the well-defined structures. Strip width decreases from PCL to PCL-CNT-5.

To obtain the detailed information of the printed scaffolds, the single window is being observed along with a grid through a bright field microscope. Moreover, several areas have been chosen in the scaffold to get the overall idea of the structure of the printed scaffolds. It is clearly observed from the images in Figure 15, as the nanoparticle percentage increases in the PCL matrix, the scaffold structure becomes more defined and ordered. The average width of PCL and its nanocomposites were determined by analysing the images through high-frequency NBS 1963A standard resolution test target (R2L2S1P1) from Thorlabs (2" x 2") and toup camera software. Figure 14b shows the graph between the average widths calculated vs the CNT percentage in the samples. The average strip widths of the PCL, PCL-CNT-1, PCL-CNT-3 and PCL-CNT-5 grid are 450 ± 30 , 380 ± 15 , 326 ± 15 , 300 ± 20 μm respectively. The distance between each horizontal and vertical stripes of the grid window increases with increasing content of CNTs. The distance between horizontal and vertical strips of the PCL, PCL-CNT-1, PCL-CNT-3 and PCL-CNT-5 grid window are 425 ± 25 , 550 ± 20 , 625 ± 20 , 675 ± 25 μm respectively (Figure 14b). The cross-junction of the grid also describes the prominent microstructure of the scaffolds. The inset of the Figure 14b shows the representative printed PCL-CNT-5 scaffold, as it is

discernible from the image that the overall scaffold structure (length, breadth) is printed in a consistent manner with high precision.

Figure 14 (a) Stereo microscopic images of PCL and its nanocomposites. (Scale Bar 2 mm)
(b) Average line width of the grid and average distance between the strips of PCL and its nanocomposites w.r.t. percentage of CNT present in the sample (Inset figure showing the representative printed picture of PCL-CNT-5 scaffold.)

Figure 15 Optical images of single window and cross junction of 3D printed pure PCL and its indicated nanocomposite grids (a) PCL (b) PCL-CNT-1 (c) PCL-CNT-3 (d) PCL-CNT-5. (Scale Bar 400 μm)

3.3.2. Characterisation of 3D printed PCL nanocomposites

Raman spectroscopic technique is being used to observe the structural integrity of CNT in the PCL matrix.

Figure 16 shows the Raman spectra of COOH functionalised CNT and their subsequent PCL nanocomposites (PCL-CNT-1, PCL-CNT-3 and PCL-CNT-5). The disordered graphite structure or sp^3 -hybridised carbons of the CNT is demarcated by the peak at 1350 cm^{-1} also known as D-band, while the structural intensity of sp^2 -hybridised carbon atoms or tangential mode is assigned to peak at 1580 cm^{-1} (G-band). The Raman spectrum of PCL shows the characteristic peaks at 1723 cm^{-1} ($\nu\text{C=O}$), $1040\text{--}1106\text{ cm}^{-1}$ (skeletal vibration), $1470\text{--}1415\text{ cm}^{-1}$ (CH_2) and $1281\text{--}1305\text{ cm}^{-1}$ (wCH_2) attributable to CH_2 groups[142, 150, 151]. Both characteristic bands (D and G) are absent in pure PCL. The PCL characteristic peaks are also discernible in the nanocomposites. As the CNT content increases in nanocomposites, the peaks attributed to CH_2 groups and skeletal vibration becomes broad due to submerging of D band and the interaction of CNT's with PCL matrix.

There is no shifting of both D and G bands after nanocomposite formation, implying only physical adsorption of PCL on the surface of CNT. The degree of functionalisation of CNTs can be estimated by the D-G band intensity ratio (I_D/I_G). The I_D/I_G ratio is calculated for CNT and its nanocomposites. The D band is merged with CH_2 group peaks of the PCL in its nanocomposites. The peaks in the range of $1200\text{--}1520\text{ cm}^{-1}$ have been deconvoluted and curve fitted. There was no significant change observed in I_D/I_G ratio for CNT and its nanocomposites, referring a physical adsorption of PCL onto CNT walls in the nanocomposites. Moreover, the structural integrity of CNT walls was totally conserved after the formation of nanocomposites.

Figure 16 Raman spectra of pure MWCNT, PCL and its indicated nanocomposites

3.3.3. Thermal behaviour

Figure 17a shows the representative DSC traces of PCL and its CNT nanocomposites. Distinct endothermic melting peaks (T_m) at 63.5, 64.3, 65 and 64.8 °C for PCL, PCL-CNT-1, PCL-CNT-3 and PCL-CNT-5, respectively were observed. Heat of fusion (ΔH) increases in nanocomposites in the presence of CNTs (73.4, 76.5, 78.5 and 79.6 J/g for PCL, PCL-CNT-1, PCL-CNT-3 and PCL-CNT-5, respectively) due to enhanced interaction between polymer chains and CNTs (Figure 17b). The enhancement in the enthalpy of fusion is probably due to the nucleation effect of CNTs. The peak temperature and enthalpy of fusion for the second run has been compiled in Supplementary Figure 2, shows the same behaviour as the 1st run. The cooling curves reveal an increase in crystallisation temperature (T_c) for nanocomposites as compared to pure PCL. The crystallisation peaks are slightly broader for nanocomposites as compared to pure PCL (Figure 17c). The gradual shifting of T_c is being noticed from 25, 33, 35 and 35 °C for PCL, PCL-CNT-1, PCL-CNT-3 and PCL-CNT-5, respectively and

nanocomposites also seems to be slightly crystalline in cooling period as compared to pure PCL (ΔH_c) 49, 51.8, 52.5 and 53.8 J/g for PCL, PCL-CNT-1, PCL-CNT-3 and PCL-CNT-5, respectively) (Figure 17d). This is presumably due to the nucleating effect of CNT on PCL crystallisation[140]. Moreover, increase in the crystallisation is also noticed for other polymers like PU due to the alignment of chains in the domains, due to self-assembly and increasing hard segment content[152]. Similarly, polymer chains may also align due to nucleation effect of CNTs, which increases the crystallisation behavior of nanocomposites[140, 153]. The increasing content of CNTs further increases the crystallisation temperature and broadens the crystallisation peak, indicating restricted mobility of polymer chains in the nanocomposite.

Figure 17 DSC thermograms of PCL and its indicated nanocomposites (a) heating curves (b) Graph representing the change heat of fusion (J/g) and melting temperature w.r.t. CNT percentage (c) cooling curves (d) Graph representing the change heat of crystallisation (J/g) and melting temperature w.r.t. CNT percentage in the sample.

3.3.4. Nanoindentation (mechanical response of scaffolds) and Conductivity

Figure 18a shows the load vs indentation depth representative curve for PCL and its nanocomposites obtained through nanoindentation[154-156]. The indentation depth is fully recovered for PCL and its nanocomposites during unloading, demonstrating the elastic behaviour of PCL and its nanocomposites even after the incorporation of CNTs. The lower indentation depth with the higher peak load is being observed with increasing CNT content in the nanocomposites. It is probably due to the increase in strength achieved via CNT reinforcement which resists the elastic deformation of the nanocomposites[154]. The representative curve of different samples with maximum load value has been shown in Figure 5a. The maximum peak load value is 1.16, 1.21, 1.27, 1.34mN for PCL, PCL-CNT-1, PCL-CNT-3 and PCL-CNT-5 respectively while the calculated average load value is 0.88 ± 0.28 , 1.12 ± 0.09 , 1.17 ± 0.10 , $1.27 \pm .07$ mN for PCL, PCL-CNT-1, PCL-CNT-3 and PCL-CNT-5, respectively (Figure 18b). The average load value and maximum peak load value gradually increase with increasing CNT content is because of the reinforcement effect of stiffer CNTs in the PCL matrix.

The elastic modulus (E) of PCL and its nanocomposites is being calculated by PCL-CNT-5 nanocomposite has an average value of 0.87 ± 0.10 GPa, which is a 70% improvement ($p=0.0014$) over PCL ($E=0.51 \pm 0.18$ GPa). PCL-CNT-1 and PCL CNT-3 also shows improvement of 35% and 55% in the E value respectively, as compared to PCL. PCL-CNT-1 and PCL-CNT-3 has average E values of 0.69 ± 0.15 ($p=0.1098$) and 0.79 ± 0.06 GPa($p=0.0102$), respectively. The average E value of PCL-CNT-5 nanocomposite is slightly higher than PCL-CNT-1 and PCL-CNT-3. Similarly, hardness is also being calculated for PCL and its nanocomposites. The value of hardness is 0.057 ± 0.010 , 0.069 ± 0.010 , 0.067 ± 0.004 , 0.072 ± 0.003 GPa for PCL, PCL-CNT-1, PCL-CNT-3 and PCL-CNT-5, respectively. The increase in hardness can be explained by the reinforcement of CNT in PCL matrix with its increasing content. Thus, the elastic modulus, peak load value and hardness increase with increasing CNT content.

Conductivity of PCL-CNT nanocomposites increases with increasing CNT content. The conductivity value of PCL^[157] is less than 10^{-15} S/cm, which increases six orders of magnitude for PCL-CNT-1. Eight and nine orders increase in the conductivity is being discerned for PCL-CNT-3 (2.2×10^{-7} S/cm) and PCL-CNT-5 (1.2×10^{-6} S/cm), respectively.

Figure 18 Nanoindentation of PCL and its indicated nanocomposites (a) Representative Maximum Load vs. displacement graph (b) Bar graph representing the comparison of hardness, modulus and average load.

3.3.5 Enzymatic Biodegradation

Biodegradation studies[140, 158] have been carried out on PCL and its nanocomposites at 37°C in *Pseudomonas* lipase type XIII enzyme media in buffer solution shown in Figure 19a. The enzyme degradation rates of nanocomposites are slower as compared to pristine PCL. The weight loss of 70 and 80% has been observed for PCL-CNT-3 and PCL-CNT-1 scaffold, respectively after 24h of enzymatic degradation against the maximum weight loss for pure PCL. The PCL exhibits the faster degradation rate as compared to nanocomposites due to greater amorphous zone as compared to the nanocomposites, which are more susceptible to hydrolysis by enzymatic biodegradation. The morphology observed in SEM images of PCL and its CNT nanocomposites have been presented in Figure 19b to compare the degradation rates. The degradation images of scaffolds have been taken after 6h. PCL and its nanocomposite scaffold having 6 grid windows for each sample is being displayed by SEM images at time $t=0$. As it is discernible from the SEM images after 6h, highest degradation is being observed for PCL followed by PCL-CNT-1 and PCL-CNT-3. Morphology of PCL and its nanocomposites cannot be observed after 24h because it was difficult to recover the samples from the enzyme solution. The degradation rate is dependent on surface area and scaffold structure. In the case of grids, the effective area and window pores enhance the binding activity and sites for *Pseudomonas* enzyme, which results in the higher and faster rate of biodegradation. However, enzymatic biodegradation of PCL and its nanocomposites can be tuned by varying the CNT percentage in the nanocomposites as well as the concentration of enzymes.

Figure 19 (a) Percentage weight loss of PCL and its indicated nanocomposite scaffolds during enzymatic (lipase from *Pseudomonas* sp., Type XIII) degradation at 37 °C. **(b)** SEM images of PCL and its indicated nanocomposites before and after degradation. The ‘0h’ and ‘6h’ indicates the time before and after 6h degradation respectively.

3.3.6. Cytotoxicity assay and cell compatibility

MTT assay was performed to evaluate both the viability and cell metabolic activity of H9c2 mouse myoblast cells on PCL and its nanocomposite scaffolds for 4 days as shown in Figure 20a. MTT assay showed that cells were not only viable on PCL scaffold but also on the PCL-CNT nanocomposite as well. Based on this experimental condition, it seems that H9c2 cells on the PCL-CNT-1 scaffold have a greater metabolic activity than those growing on the PCL or PCL-CNT-3 scaffold. Statistical analysis of the data showed that there is a significant difference for PCL-CNT-1 scaffold ($p=0.0087$) when compared to the control, indicating that the PCL-CNT-1 scaffold may provide more favourable conditions for the adhesion and proliferations for the H9c2 cells than PCL-CNT-3 scaffold. One major concern with the use of CNTs is toxicity to the cells due to the use of chloroform[159]. Moreover, the cell scaffold fluorescence images were also captured to observe the cell adhesion after 4 days; cells are adhered on the surface of scaffolds and were growing in a healthier way. The cell proliferation was observed in all the scaffolds but was more in PCL-CNT-1 as compared to other scaffolds (Figure 20b). Additional assay of the PCL–CNT scaffolds using live/dead staining assay for 4 days shown in Figure 21 was performed to show that cells were growing healthy with both PCL and its nanocomposites. Although it has been mentioned that CNT improves the proliferation of the cells[159], it was observed in other cells types like L929 and NIH3T3 cells in PCL matrix[158]. The slight increase in proliferation of H9c2 myoblast cells on PCL-CNT nanocomposites was observed. No cytotoxic effect on H9c2 myoblast cells due to added CNTs was shown after several days in the printed PCL–CNT scaffolds as most cells can be seen attached to the scaffold and were alive based live/dead staining and MTT assay.

Figure 20 (a) MTT assay of H9C2 myoblast cells in PCL and its indicated nanocomposite scaffolds. (b) Fluorescence images of H9C2 myoblast cells obtained through dye staining after 4 days of growth on PCL, PCL-CNT-1, and PCL-CNT-3 scaffolds. (Scale Bar 100 μ m)

Figure 21 Fluorescence images of live (green) and dead (red) H9C2 myoblast cells obtained through dye staining after 3 days of growth on (a) Control (b) PCL, (c) PCL-CNT-1, and (d) PCL-CNT-3scaffolds. All the images are at a magnification of 10X.

3.4 CONCLUSION

Fabrication of well-defined PCL and its CNT composite scaffold structures was achieved by 3D printing. Incorporation of CNTs reinforced the alignment of the polymer chains resulting in slight enhancement in crystallinity and interaction with PCL matrix, which was observed from DSC and Raman spectroscopy. Nanoindentation results have shown the gradual enhancement in elastic modulus, hardness and maximum peak load in PCL-CNT composites. Enzymatic biodegradation revealed the structural and nanotube content dependence on the rate of biodegradation. Biodegradation can be tuned by selecting particular scaffold structure followed by the CNT content and enzyme concentration. MTT assay and fluorescence imaging results have shown that the myoblast cells are attaching to the scaffolds and are in healthy condition after 4 days. It can be concluded that PCL-CNT nanocomposites with 1% CNT show the optimal conductivity and stiffness for the proliferation of H9c2 cells. To conclude, PCL scaffolds have been fabricated with different CNT content using the 3D printer. Tuned degradation and cell compatibility might be further utilised for the application of the scaffolds in tissue engineering, as the PCL-CNT matrix can be enzymatically biodegraded after the formation of cardiac tissue.

CHAPTER 4: HONEYCOMBED STRUCTURES FOR CONTAINMENT OF LIQUID BACTERIA CULTURE FOR QS SENSING

4.1 INTRODUCTION

Microbiology is the study of a huge and diversified group of microorganisms such as bacteria and virus that are considered to be individual organisms[160]. Microorganisms not only adhere but colonise mostly on all materials surfaces[161]. In the medical community, there is currently an increasing awareness towards the polymicrobial interactions which are linked to health and diseases[14]. Polymicrobial infections are associated with two or more microorganisms. For a better understanding on how a polymicrobial infection works, microbe-microbe interactions have to be studied, however, a suitable model is required[162]. It is widely believed that most bacteria in our nature aggregate as biofilms, a form whereby they behave very diversely[163].

Polymicrobial biofilm communities can be defined as a complex environment housing a varied collection of microorganisms (fungi, bacteria, and viruses) on a biotic or abiotic surface and encapsulated in a self- and/or host-derived hydrated matrix, comprising of polysaccharide[14]. These environments provide protection against protozoan predators[166] and host immune defences[167] as well as heighten resistance to antibiotics[168]. The growth and adherence of bacteria into biofilms on diverse range of surfaces impact the ecosystems[169] and medical industry. By eradicating biofilms on medical devices, improvement in human health as well as possible financial incentives can be observed. This encourages more research to be done on the mechanisms of biofilms formation and development as well as techniques to remove and prevent them[170]. One method of studying the biofilm formation is through the use of a model organism called *Pseudomonas aeruginosa*[171].

One-way bacteria communicate with each other is through Quorum sensing(QS)[164]. *Pseudomonas aeruginosa* is a well characterised QS bacteria. The prevalence of this organism as well as its pathogenic impact on the medical field has made it an important bacterium to study on. Furthermore, the increase in the cell's resistance towards antimicrobial therapy due to the formation of biofilms led people suffering from cystic fibrosis to have acute chronic infections[165]. Currently, the most common way to study the QS between communities of *Pseudomonas aeruginosa* is through the use of confined hydrogel chambers or a scaffold[172].

Current fabrication of these hydrogel chambers used soft lithography is not only slow but also requires the manual creation of a PDMS mould, incurring additional cost. Given the endless need for high efficient fabrication to hasten of these hydrogels scaffolds for more studies to be done, a faster and more efficient alternative production method is required.

3D printing has gained much popularity due to its ability to print complex structures without the need for additional tooling, making it a good alternative for creating scaffolds in the field of biology. Stereolithography is a UV based system that crosslinked photopolymer resin layer by layer for creating the 3D structures. The photopolymer material Poly (ethylene glycol) diacrylate (PEGDA) was chosen due to its non-toxicity, ease of use and cytocompatibility[173]. Another key property of PEGDA is the ability to reduce bacteria adhesion and resist the adsorption of mammalian cells and proteins[174]. Thus, this characteristic allows biofilm to develop in printed gels without attaching to the walls and top surface of the hydrogel scaffold, allowing the growth of free standing biofilms in the hydrogel scaffold. Furthermore, crosslinked PEGDA polymers can be tuned for different applications by using different ratios of PEGDA monomers and changing the processing condition during photopolymerisation [175]. This is beneficial to achieving a final biomaterial or structure with a desired mechanical characteristic.

For this chapter, 3D porous structure for containment of liquid bacteria for studies on *P.aeruginosa* QS was fabricated with the Asiga PICO2 3D printer. Optimisation and printability of PEGDA with Igracure 819 had to be research on as it was not used with this commercial printer before. the. By tuning the different concentrations of Igracure 819, well-defined dog-boned and honey combed structures were designed and fabricated. Characterisation on the printed structures in terms of mechanical properties and swelling ratio and its compatibility with bacteria were performed. Finally, honeycomb chambers were used with different *P. aeruginosa* species for studies on diffusion of the QS molecules.

4.2 MATERIALS AND METHODS

4.2.1 Material for printing

Poly (ethylene glycol) diacrylate (average M_n 700) (**455008**) and Phenylbis (2,4,6-trimethylbenzoyl) phosphine oxide **BAPOs** (**511447**) also known as Igracure 819 were purchased from Sigma Aldrich Chemistry. *Pseudomonas Aeruginosa* (*P. aeruginosa*) strain PAO1-GFP and PAO1-LSB-GFP was kindly donated by Prof Kimberly Kline at SCELSE. Bacteria were grown in Luria-Bertani (LB) broth, cultured at 37°C under static conditions overnight. Overnight cultures were normalised to $1-2 \times 10^8$ CFU/mL in PBS to OD600 nm (optical density) 0.4 prior to dilution for QS assay.

4.2.2 Preparing solution for 3D Printing

Concentration	Igracure 819	PEGDA
0.1% Igracure 819	0.02g	20 ml
0.5% Igracure 819	0.1g	20 ml
1.0% Igracure 819	0.2g	20 ml

Table 2 Measurement of material for making PEGDA solution

The quantity of PEGDA and Igracure 819 required is shown in Table 2. Different amounts (0.1%, 0.5% and 1%) of Igracure 819 powder were determined according to w/v% of PEGDA was slowly added into the PEGDA solution with stirring. The contents were stirred continuously for approximately 45 to 60 min to ensure that Igracure 819 was fully dissolved. Once the mixture has become a homogenous solution, it is ready to be used for printing.

4.2.3 Printing of Scaffold

A dog-boned flat specimen and the honeycomb structures were modelled on computer-aided design software called Solidworks®. Dog scaffolds were designed as per ASTM D639-98

standard while honeycomb structures approximately 18.6mm X 11.3mm X 40mm and printed using the Asiga Pico 2 3D printers which uses a 405nm LED lamp with slight modifications.

To print PEGDA, a modified build tray was printed to accommodate small amounts of PEGDA solution. Once the tray is fixed and calibrated with the build stage, approximately about 2ml of PEGDA solution was used to fill up the tray. The scaffolds design was converted to STL. file and sent to the printer. Structures were photopolymerised with an exposure time of 1 sec for each layer with a z-resolution in 0.025mm and light intensity approximately 18.0mW/cm². Once the structures have been printed, it was rinsed with isopropanol and then post cure using a UV lamp(405nm) for another 6 mins to obtain the final structures.

4.2.4 Tensile Test

The elastic module was performed with the Instron Microtester Model 5547 (20mm gauge length). For each different Igracure819 concentration, a dog-boned structure was printed and this experiment was repeated at least four times. 819 concentration were tested. 100N load cell was used and testing were performed accordingly to the manufacturer manual. A second tensile test was performed after samples were being subjected to swelling. Firstly, the different concentrated PEGDA dog-boned samples were each placed into centrifuge tube containing 20ml of Phosphate-buffered Saline (PBS). The samples were then left in the incubator at 37⁰C for 24hrs. The samples were removed from the centrifuge tubes, carefully patted dry with tissue to prevent slippage at the clamps and then observed. After which, they were subjected to the same uniaxial test using the Instron machine. The next few steps were then repeated with reference to the previous tensile test done with the dry samples.

4.2.5 Swelling Test

A total of six PEGDA hydrogel samples, two of each Igracure 819 concentration were first weighed and then placed into a centrifuge tube containing 10ml of LB medium and TSB medium. The samples were kept at 37°C in the oven to simulate the growth of bacteria. After 24h, the samples were removed from the centrifuge tube, carefully patted dry with tissue to remove the medium on the surface of the hydrogels and then weighed. The hydrogels' mass swelling ratio (Q_m) was then determined using the equation $Q_m = (W_S - W_0) / W_0$ whereby W_0

is the dry weight and W_s is the swollen weight of the hydrogel. A bar chart of the Q_m for all the different Igracure concentration of samples was plotted.

4.2.6 Stereo Microscopy

The Olympus SZX7 Stereo Microscope for observation of the printed scaffold. A total of eight different parts of the scaffold were captured. These eight images would then be reconstructed to form a single image of the whole piece of the scaffold for analysis. At the same time, the image of the whole piece of scaffold was taken with a camera. The main segment of interest of the scaffolds are at the internal edges of the hexagon as well as the wells.

4.2.7 Scanning Electron Microscopy (SEM)

Scanning electron microscopy JEM-5600LV (SEM) test was used to observe the surfaces of the specimens for analysis. Samples were gold plated by using the JEOL-165C gold sputtering machine at 20kA with 30 seconds of plating. The scaffolds of different concentrations were observed under the SEM.

4.2.8 *Pseudomonas Aeruginosa* Culture & Observation

A trial experiment to study the QS between communities of *P. aeruginosa* was carried out. To prepare the bacteria colony, *P. aeruginosa* was first streaked out onto an agar plate containing LB broth. The agar plate was then kept inside 37°C incubator overnight. Next, the individual bacteria colony was picked and inoculated in 10ml LB broth after which the setup was left under static condition of 37°C overnight.

Before observation for fluorescence could be done, bacteria cultures were first normalised in PBS to achieve OD_{600nm} (Optical Density) 0.4. Then, 150µl of LB broth and 10µl of normalised cells was added into each well of the culture plate and observed at different time points to determine the diffusion of the molecules until the bacteria starts to fluoresce. Honeycomb structures were created for testing.

4.3 RESULTS AND DISCUSSION

4.3.1 Tensile Test for Dry Dog-boned samples

The purpose of the tensile test is to determine the tensile modulus, modulus of toughness as well as the strength at fracture of the PEGDA dog-boned samples with different Igracure 819 concentrations. Using the Bluehill software that is provided by Instron, the raw data was exported and plotted on OriginPro shown in Figure 22 for analysis.

Figure 22 Tensile Stress-Strain Curve for Dry Samples. Black = 0.1% Igracure 819, Red = 0.5% Igracure 819 and Blue = 1% Igracure 819.

Figure 22 shows the maximum tensile stress that the dog-boned samples are able to withstand before it started to break. All the samples showed no deformation through the tensile extension unless sufficient amount of load was applied. There is a linear relationship between the increasing tensile stress and tensile strain, implying the samples were withstanding an increasing load before starting to fail. This linearity continues till it reached a sudden drop in the tensile stress-strain curve, which is characteristic for PEGDA hydrogel due to it relatively

brittle nature. This drop also implies that the dog-boned specimen has failed and fractured as it is no longer able to withstand further extension.

The average maximum tensile stress before failing for 0.1% Irgacure 819 samples (black lines), 0.5% Irgacure 819 (red lines) and the 1.0% Irgacure 819 samples (blue lines) were approximately 2.65 MPa, 1.97 MPa and 1.43 MPa respectively. This indicates with lower concentration of Irgacure 819 there was a higher the tensile strength of the PEGDA hydrogel. With the increasing tensile strength, this also means that the samples are getting less brittle.

The tensile or Young's modulus of the samples increases with increasing concentration of Irgacure 819 which is shown by Figure 23. Since tensile modulus measures an object's resistance to elastic deformation, a higher value would mean that the samples are getting stiffer. Furthermore, tensile modulus is also a direct measure of the material's strength[176].

Figure 23 Tensile Modulus Chart for Dry Samples

There was a significant jump of approximately 0.8 MPa in the average tensile modulus between the 0.1% Irgacure 819 samples to 0.5% Irgacure 819 samples. Subsequently, the average tensile modulus only increased about 0.4 MPa between the 0.5% Irgacure 819 samples and 1.0% Irgacure 819. This seemed to suggest that increasing concentration of Irgacure 819 does not have much of an effect on the crosslinking of PEGDA since it is reaching optimal material strength.

The results obtained from tensile test of the dry dog-boned samples implied that by using a higher concentration of Irgacure 819, a more brittle yet stiffer hydrogels would be obtained. It is also understood that a larger amount of cross-linking agent or photoinitiator would result in a higher cross-linking density in hydrogels which also meant that more permanent bonds were formed. More permanent bond form would imply an increase in strength but a reduction in elasticity. As such, it can be concluded that the mechanical properties of the hydrogel are highly dependent on the cross-linking density and that material strength can be increased by adding more amount of photoinitiator.

4.3.2 Tensile Test for Swelled Dog-boned Samples

The purpose of the second tensile test was performed to provide a better understanding on how the hydrogel may behave when subjected to swelling. Firstly, the different Irgacure 819 concentration PEGDA dog-boned samples were each placed into centrifuge tube containing 20ml of Phosphate-buffered Saline (PBS). The samples were then left at the incubator at 37°C for 24hrs to simulate the diffusion test. Samples were then patted dry before running the tensile test. Figure 24 showed the maximum tensile stress that the swelled dog-boned samples are able to withstand before they fail. Some of the samples were deformed or cracked before undergoing tensile testing which resulted in a reduction in the sample sizes.

Figure 24 Tensile Stress-Strain Curve for Swelled Samples

There is a similar trend when compared to dry samples for the tensile stress-strain curves except for a few anomalies. Thus, indicating that the nature of the fabricated samples remained.

However, there is a drop in the maximum tensile stress for all the samples. The average maximum tensile stress that the 0.1% Igracure 819 samples (black lines) could withstand before failing dropped to approximately 2.06 MPa. The average maximum tensile stress that the 0.5% Igracure samples (red lines) can withstand before failing, dropped to about 0.82MPa. While the average maximum tensile stress that the 1.0% Igracure 819 samples (Blue lines) could withstand before failing, dropped to approximately 0.63MPa. This indicated that when samples are subjected to swelling, they end up becoming more brittle.

One of the sample from 1.0% Igracure 819, the curve increased linearly at the beginning but started to plateau off at about 0.2 MPa. After that, no further drop could be seen. The reason for such an occurrence is probably due to the slippage of the sample from the clamp. Given

that the samples were manually clamped one by one, human error is more inevitable which resulted in the slipping of samples as they were not properly gripped at the clamps.

There were also multiple sudden drops observed for all three different concentrations samples. One explanation for this phenomenon is that the swelling of the samples might have resulted in delamination and wrapping. The multiple sudden drops in the tensile stress-strain curves reflect the delamination between the 3D printed layers, as there are build layer by layer.

Table 3 shows some the images of the unused swelled dog-boned samples due to their deformity.

	0.1% Igracure 819	0.5% Igracure 819	1.0% Igracure 819
Side View
Top View

Table 3 Images of unused and damaged dog-boned samples

Table 3 shows the dog-boned samples which were damaged due to lamination. This is probably due to the hydrogel water swelling nature. The swollen structures mean that it has permanent bonds between the polymer chains, resulting in inability to disperse[177]. After absorbing certain quantity of PBS, further swelling of the samples occurs, causing deformation in the network chain. This deformation is then seen as bents and cracks in the samples. Furthermore, it can be observed that 1.0% Igracure 819 sample have the most cracks since a higher

concentration of Igracure 819 would mean a higher degree of cross-linking resulting in more permanent bonds formed.

4.3.3 Swelling Test

The purpose of the swelling test is to investigate the percentage swelling by various Igracure concentration with PEGDA hydrogel in Luria-Bertani (LB) media and Tryptone Soya Broth (TSB) media.

The swelling test indicated that the 0.1% Igracure 819 hydrogel scaffold has a swelling ratio of 1.22 and 0.35 for TSB medium and LB medium respectively whereas the 0.5% Igracure 819 hydrogel scaffold has a swelling ratio of 0.32 for both TSB and LB medium. 1.0% Igracure 819 hydrogel scaffold has a swelling ratio of 0.22 and 0.31 for TSB medium and LB medium respectively. As such it can be determined that higher Igracure 819 concentration would result in a decrease in the degree of swelling.

Figure 25 Swelling Ratio Bar Chart 0.1%, 0.5% and 1.0% Igracure concentration hydrogel scaffolds soaked in TSB medium and LB medium

Figure 25 shows the swelling ratio of the 0.1%, 0.5% and 1.0% Igracure concentration hydrogel scaffolds soaked in TSB medium and LB medium. As mentioned before, the swelling ratio of

0.1% Igracure 819 hydrogel scaffold soaked in TSB medium is at an unusually high value of 1.22. This is because during soaking, the hydrogel scaffold has already fractured into two pieces as displayed in Figure 26. As such, this might lead to greater absorption of the TSB medium, given the higher surface exposed area which leads to more swelling.

Figure 26 Fractured Scaffold Due to TSB Swelling

It can also be observed that the swelling ratio varies for the two-medium regardless of the concentration of Igracure 819 in the hydrogel scaffold. This shows that degree of swelling can be controlled by external conditions which are in this case the type of swelling agent. As stated, more swelling is equivalent to a higher diffusion rate. The result of the swelling test thus demonstrated that it is possible to increase diffusivity of the hydrogel through reducing the Igracure 819 concentration.

4.3.4 Stereo Microscopy Images

The purpose of the microscopy test is to determine the printability of the printed scaffolds with 0.1% Igracure 819, 0.5% Igracure 819 and 1.0% Igracure 819. Printability refers to the resolution of the printed parts. The images of the sterile microscopy test for the PEGDA scaffold containing different concentrations of Igracure 819 were summarised in Table 4 followed by observation and analysis to investigate the printability of the scaffolds.

	0.1% Igracure 819	0.5% Igracure 819	1.0% Igracure 819
Overall Photo
Reconstructed Photo
Segment A
Segment B
Segment C

Table 4 SEM images for the second degradation test

The images showed that all the different Igracure 819 concentrated structures are printed quite accurately. Based on the ruler placed below the scaffolds, the width of the scaffold is about 1.86cm. The size of the printed scaffold matches the initial Solidworks CAD model that was initially designed thus showing the accuracy of the DLP printer, PICO2, used.

However, from the reconstructed photo, deformities were observed for the 0.1% and 0.5% Igracure 819 hydrogel scaffold. This differs with the printed 1.0% piece of hydrogel scaffold which showed a nice honeycomb structure with no deformities. This is because hydrogel fusion

has occurred during printing[30]. The occurrence is probably due to low degree of crosslinking or in other words lack of photoinitiator.

From segment A and B, the corners of the hydrogel scaffold can be observed more closely. It can be observed that deformities in the form of flashes were present at the corners of the scaffold for the 0.1% and 0.5% Igracure concentration. Given that the corners being the smallest adjacent intersection point, it is more probable for hydrogel diffusion to occur because of the effect of area overlapping.

Segment C shows a clearer image of the wells of the scaffolds. The well of the 0.1% Igracure concentration hydrogel scaffold is filled with excess cured resin indicating bad resolution in printing. The presence of this excess cured resin is probably a result of collapse due to material instability caused by very low degree of crosslinking[177].

The result of the sterile microscopy test showed that the concentration of 819 used must be at least 1.0% to achieve a high resolution and accurate printing. Any amount lower would only result in the appearance of deformities such as flashes or excess cured resin.

4.3.5 Scanning Electron Microscopy (SEM) Test

The purpose of the scanning electron microscopy (SEM) test is to observe the surfaces of the specimens for analysis. The results of the SEM test for the hydrogel scaffolds were summarised in Table 5.

	0.1% Igracure 819	0.5% Igracure 819	1.0% Igracure 819
x50 0° Centre			
x150 0° Surface			
x150 45° Surface			
x500 45° Surface			
x50 45° Inner Wall			

Table 5 SEM Images for Scaffolds

All the different Igracure 819 samples have the same mesh like pattern (x150 0° Surface) observed on the surface of the scaffold. This mesh like pattern is more defined for 0.1% Igracure 819 scaffold as compared to 0.5% and 1.0% Igracure 819 scaffolds.

From the 45° tilted images, it can be observed that all the mesh like surface turns out to be more of like dotted like surfaces. These dotted like surface seems to suggest that all the scaffolds have a rough surface.

However, from the x500 45° tilted images, it seems that the dotted like surface for 0.1% Igracure 819 scaffold is coarser as compared to that of 0.5% and 1.0% Igracure 819 scaffolds. As such, this seems to suggest that lower amount of photoinitiator would produce a rougher surfaced scaffold which is favourable, given that a higher roughness surface would promote better bacteria adhesion[178].

The inner wall images with a focus of x50 45° shows that the inner walls of 0.1% Igracure 819 scaffolds was not printed accurately as layering could not be observed. It can also be noticed that there is a difference in the layering pattern between 0.5% Igracure 819 sample and 1.0% Igracure 819 sample. There is an obvious change in the orientation in the layering pattern in 1.0%, from that of horizontal to vertical. This might be because of the outermost surface of 1.0% Igracure 819 sample being eroded by the isopropanol thereby revealing the inner horizontal layer during the cleaning task of the post processing step since the tendency to damage a part for this step is particularly high.

4.3.6 Summary of Results

The results of the experiments conducted proved that the concentration of Igracure 819 used could significantly affect the mechanical properties, the printability, as well as diffusivity of PEGDA hydrogels.

Although lower Igracure 819 content brings about better diffusivity, 0.1% and 0.5% Igracure 819 scaffold were not able to get a desirable printing resolution and accuracy with the DLP printer PICO2. At the same time, a higher Igracure 819 concentration would be preferred as it results in a better material strength and good printing resolution. The printing of 0.1% and 0.5% Igracure 819 scaffolds showed deformities such as flashes or excess cured resin. Thus, it is not possible to be a scaffold for the study of the QS between communities of *P. aeruginosa* as deformities will affect the scaffold ability to contain the required amount of *P. aeruginosa* cells. As such, 1.0% Igracure 819 scaffold was used for the next part of experiment.

4.3.7 *Pseudomonas Aeruginosa* Culture & Observation

Bacteria was tested using 8 well chambers made from plastics which prevents the diffusion of the QS molecules to act as a control. At the same time to ensure that the *P. aeruginosa* strains can be detected, it was grown in *P. aeruginosa* supernatant to ensure it can fluorescent. However, when *P. aeruginosa* was seeded into the PEGDA hydrogel scaffolds, some issues surfaced and sadly, no result for QS sensing could be obtained for this test and more work will be required to obtain the result.

Firstly, the hexagon structures printed onto the glass slide or coverslip were not strongly attached so when they were seeded with the bacteria culture, the hexagon structures detached from the glass during experimentation. This resulted in the spillage of bacteria culture. Two methods were explored to resolve this issue: (1) by washing the glass with piranha solution and salinising it or (2) using araldite glue. However, due to time constraint, only araldite glue was able to be experimented on as materials for making piranha solution did not arrive in time. Araldite glue is commonly used to bond tubing to microfluidics device[179] since it is non-toxic to cells. However, when it was applied to the printed PEGDA, it could not attach properly to the glass slide. This could be due to the surface of the printed PEGDA. Salinisation of the glass slides should work as it was shown previously with PEGDA, it seem to attach better to the glass slides[180].

Next, due to the small amount of media, evaporation occurs and constant adding of solution is required. So, in order to prevent it, structures have to be either enclosed in a moist zip lock bag or be surrounded to prevent media from being absorbed. Finally, cell density may have to be optimised to ensure high concentration of secreting AHLs to go beyond the threshold of the biosensor's sensitivity though this occurrence is highly unlikely[181].

4.4 CONCLUSION

In this research, three different types of Igracure 819 concentrations, 0.1%, 0.5% and 1.0%, were used to form hydrogel structures that have different swelling, mechanical properties as well as printability. The hydrogel structure made with 1% Igracure 819 concentration swelled less, was stiffer but more brittle when compared to the other hydrogel with lower concentration of Igracure 819. As such, a proper design of printing parameters and concentration of Igracure 819 was obtained for this reserach. Printability or well-defined structures with ease of post processing was obtained with 1% Igracure 819 concentration. Initial results showed that with increasing Igracure 819 concentration, the stiffer and more brittle the PEGDA hydrogel become, smaller swelling ratio is obtained but there is a significant decrease in terms of printability as only hydrogel scaffold with 1.0% concentration of Igracure 819 could print without any observable deformities. Given that no observable result was for the bacteria culture, a re-design or change of material for the scaffold might be needed. Factors such as the working distance of the microscope lens, adhesive strength of biomaterial and colour of the materials should be taken into consideration in order to improve the observation set up. Although, bacteria interaction could not be shown in this current scaffold, with more time, a workable PEGDA scaffold for cell interactions studies can be obtained.

CHAPTER 5: HYDROGEL STRUCTURES FOR STUDIES OF *E. coli* and *S. aureus* interactions

5.1 INTRODUCTION

Urinary tract infections (UTIs) is one of the commonly acquired bacterial infections in community and hospitals[182]. There is an estimated annual occurrence of well over 8 million UTIs in The United States of America alone[183]. The bacteriology of UTI is very predictable: although there are several different species which cause UTI, majority are caused by Gram-negative, facultative anaerobic, uropathogenic *E. coli* (uPeC). Other species such as streptococci, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, *Citrobacter freundii*, *Enterobacter cloacae*, and *Staphylococcus aureus* are also accounted for with culture based methods. Bacteria flourish as 3D structured polymicrobial biofilm communities within the human body [184]. They adapted multiple physical and chemical cues such quorum sensing, sporulation to aid in their communication with one another[184]. The important molecular factors involve in bacterial fitness and pathogenicity (e.g., oxygen, QS signals) are believed to vary according to the size and density of the bacterial aggregates[184]. Spatial organisation of bacterial aggregates in the biofilm may contribute in facilitating community attributes, altering virulence of infections or defending against external environment, ie antibiotics[184].

To explore the behaviour of spatial organised bacteria interactions, various microfabrication technologies including microfluidic devices, microcavities, and ultralow-volume liquid droplet are used[180, 185-188]. These approaches have been useful for limiting physical attributes of microhabitats in terms of size and shape. However, none can define the specific 3D geometry of bacterial aggregates or patterning of multiple populations in the microscale. One possibility is to create microscopic 3D bacterial chambers from direct laser writing[189] with a highly soluble protein bovine serum albumin (BSA)[190, 191]. By using a femtosecond laser instead of traditional laser, it enables longer wavelength to possess a similar electronic excitation like a single photon which requires much higher energy[86, 87]. This nonlinearity not only excites the photoinitiator molecules with less energy but material cross-linking can occur within the immediate vicinity of the focal volume[88]. As a result, picoliter-sized BSA chambers also known as “lobster traps” was fabricated using 2PP that can confine individual motile bacteria and allow bacteria to grow into clonal populations which cannot be observed in batch culture[190]. The porous BSA structures allow diffusion of biologically relevant molecules (ie QS signals and antibiotics), providing researchers another avenue for investigating bacterial

social interactions based on the entrapment shape, bacteria population density and flow rate of media through the chamber.

The interactions between two different bacteria species *E. coli* and *S. aureus* will be explored in this chapter. It was initially observed that there was a reduction of *S. aureus* when it was grown in close proximity with *E. coli* through proximity and mixed microcolonies assay. To determine the predatory mechanism, RNA sequencing and a mutant transposon library was created and analysed. Interestingly, when *E. coli* and *S. aureus* were observed in the macroscale, the predatory effect was not observed but when *E. coli* was mixed directly with *S. aureus*, the predatory effect was observed. This could mean that for the predatory effect to be seen, both species have to be placed in close proximity. To gain a deeper understanding on how the spatial distance between each bacterium might affect its interactions, two photon polymerisation was performed on a solution composed of gelatin and a photosensitising molecule (rose Bengal) that promotes chemical cross-linking of polypeptides following photoexcitation. Well defined structures of individual or mixed species were created and observed under a confocal microscopy to determine their interactions. The fluorescence intensity of the two fluorescence human pathogens, Gram-positive *S. aureus* and Gram-negative *E. coli*, will be used to determine their growth.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial Strains and Growth Conditions

Strains, plasmids, and primers used in this study are listed in Supplementary Table 2. *E. coli* UTI89 and *E. coli* MG1655 were grown in Luria-Bertani (LB) broth, while *S. aureus* (MN8, HG001, RN1, RN450 and ISP427) were grown in Tryptone Soy Broth (TSB) cultured at 37°C under static conditions. LB agar supplemented with vancomycin at 5mg/ml was used for *E. coli* selection, and TSB supplemented with colistin at 10 mg/mL and nalidixic acid at 10 mg/mL was used for *Staphylococcus* strain selection. Overnight cultures were normalised to $1-2 \times 10^8$ CFU/mL in PBS to OD_{600 nm} (optical density) 0.4 and 0.5 for *E. coli* and *S. aureus* respectively, prior to dilution for assays described below. For planktonic and macrocolony assays, bacteria were cultured at 37°C under 200 rpm or static conditions on tryptone soy broth supplemented with 10 mM glucose (TSBG) and solidified with 1.5% agar when appropriate (Oxoid Technical No. 3). Iron depletion was achieved by supplementation with 2,2D (Sigma-Aldrich). Construction of *E. coli* UTI89 mutants was performed as described previously [192].

5.2.2 Macrocolony assay

Macrocolony were performed as previously described[193] with minor modifications. Macrocolonies were produced by inoculating 5µl of normalised culture onto the surface of TSBG agar and incubated at 37°C. After 8 hours and 24 hours incubation, macrocolonies were excised and resuspended in 1ml PBS, followed by CFU enumeration by plating on selective media or RNA extraction and sequencing,

5.2.3 RNA extraction, RNAseq and Analysis

Mature mix microcolonies were cultured for 6hr on TSBG, in biological triplicate, and were subjected to total RNA isolation by bacterial cell disruption with Lysing Matrix B (MP Biomedicals) in TRIzol Reagent (Ambion). This was followed by chloroform extraction and isopropanol precipitation to purify the nucleic acids. DNase treatment (TURBO DNA-free kit, Ambion) facilitated removal of contaminating genomic DNA. Measurement of RNA concentration, quality and potential DNA contamination were quantified with Qubit RNA Assay and Qubit High Sensitive dsDNA assay (Invitrogen). Ribosomal RNA (rRNA) was depleted from RNA samples using the Ribo-Zero Magnetic Bacterial Kit (Epidemiology; Epicentre), and depleted RNA was converted cDNA using the new England biolabs NEBNext RNA First Strand Synthesis Module and NEBNext Ultra Directional RNA Second Strand Synthesis Module. The newly converted cDNA was subjected to library preparation for sequencing on an Illumina MiSeq2500 machine. Qubit RNA Assay and Qubit High Sensitive dsDNA assay (Invitrogen) were again used to quantify RNA quality and purity. A minimum concentration of 40-80ng/µl rRNA depleted RNA, DNA: RNA 1:10 maximum, and a RIN value ≥ 8.0 as measured by Agilent RNA ScreenTape (2200 TapeStation System) required QC checks prior to Illumina sequencing.

RNA sequencing reads were mapped to the *E. coli* UTI89 reference genome (GenBank assembly accession GCA_000013265.1) or the *S.aureus* HG001 (GenBank assembly accession GCA_000013425.1 (using BWA (v0.5.9) with default parameters [194, 195])). Sequencing reads (Accession number NCBI: PRJNA339105) mapping to predicted open reading frames (ORFs) were quantified using HTSeq [196]. Ribosomal sequences were filtered out of the data set. Differential expression analysis were performed in R (version 2.15.1) using the Bioconductor package, *edgeR*[196]. Significantly differentially expressed genes were determined using an FDR cutoff of 0.05 and a fold-change cutoff of 2 (either up- or down-

regulated). We annotated differentially expressed genes using a combination of KEGG annotations, as well as manual annotation using operon and other functional data from literature analysis.

5.2.4 Electrocompetent cells/Transposon Library Screen

UTI89 was made electrocompetent according to [197]. Briefly, UTI89 was grown overnight in LB medium lacking salt (no-salt LB) at 37°C with shaking. ml of overnight culture was used to inoculate 500 ml of prewarmed (37°C) no-salt LB and incubated at 37°C with shaking until it reached an A_{600} of 0.6 to 0.75, at this point the culture was chilled on ice for 15 min. Bacterial cells were pelleted at 8,000 rpm for 10 min, washed twice. First in 75 ml of ice-cold 10% glycerol and then in 50 ml of ice-cold 10% glycerol and resuspended in 1 ml of ice-cold 10% glycerol. The resulting electrocompetent cells had a transformation efficiency of 10^7 to 10^9 CFU/ μ g of DNA.

Transposon mutagenesis was performed by electroporating 260 ng of the EZ-Tn5 <R6K γ ori/KAN-2>Tnp Transposome kit (Epicentre) in 100 μ l of UTI89 electrocompetent cells according to the manufacturer's instructions (Epicentre), followed by a 60-min recovery in SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM glucose) at 37°C with shaking. The entire transposition reaction was then diluted 1:10,000 and plated in 100- μ l aliquots on LB-kanamycin plates to select for clones that contained a successful transposition event.

An *E. coli* UTI89 transposon library containing 15073 mutants were screened to give a 3X coverage. Individual colonies were picked and stocked in individual 96-well format microtiter plates. These were then cultured in 200 μ l of TSB with static overnight incubation at 37°C. Cultures were the normalised to an OD_{600nm} of 0.4 (1×10^8 CFU/ml) in PBS. A primary screening of the *E. coli* transposon library was performed by inoculating TSBG media with 1×10^5 CFU/200 μ l of normalised transposon mutant *E. coli* with 1×10^5 CFU/200 μ l of the normalised HG001-GFP culture resulting in an inoculum mix ratio of 1 Ec:1 SA. The microtiter plates were then incubated for 21 hours at 37°C statically. Fluorescence intensity of *S. aureus* was taken using the Tecan Infinite® 200 PRO spectrophotometer (Tecan Group Ltd., Männedorf, Switzerland). Fluorescence intensity results with an 50% reduction in *S. aureus* killing as compared to the wild type were validated again in the same assay. Secondary

validations of the transposon mutants were then performed with 3 biological replicates with 4 technical replicates to quantify the mutants before sending for sequencing.

5.2.5 Transposition mapping.

Transposon mutations were mapped using multiple-round PCR with primer Inv-1 (ATGGCTCATAACACCCCTTGTATTA) or Inv-2 (GAACTTTTGCTGAGTTGAAGGATCA). The resulting amplicons were purified (Qiagen) and sequenced using the KAN-2 FP-1 forward and KAN-2 RP-1 reverse primers (Epicentre).

5.2.6 Planktonic Growth Assays

Microtiter plates were inoculated and prepared as described for the biofilm assay. Planktonic growth was measured by recording the OD_{600nm} over a period of 18 hours at 37°C with the Tecan Infinite® 200 PRO spectrophotometer (Tecan Group Ltd., Männedorf, Switzerland). n=3 biological replicates with three technical replicates for all growth curve assays. For the polymicrobial assay, fluorescence intensity reading was taken after adding 8ul of normalised culture of mix species and added into the 96 well plates.

5.2.7 3D printing of gelatin microstructures

Well defined 3D structures of gelatin have been designed and fabricated via 2-photon direct laser writing using 780nm wavelength from femtosecond laser for biological applications. Custom designed setup as depicted in Figure 27 includes *Femtofiber Pro*, erbium doped ultrafast femtosecond laser from Toptica photonics, Germany; 60X objective lens for focused illumination, *PI Nano* piezo stage controller, *Nikon eclipse TS100* microscope, Thorlabs shutter controller (SC10) and LabVIEW based GUI interface. This setup has been extensively used for this laser-based lithographic technique where polymer microstructures are formed via 2-photon polymerisation due to cross linking at focal point. It is interesting to observe how the laser power affects the feature size of the fabricated gelatin structures. Variable beam-splitter/Attenuator is being used for altering the laser power for the fabrication.

Figure 27 Fabrication of 3D microstructures through 2-Photon direct laser writing

For printing of gelatin structures, initial printing was done according to Shear[184] before changing the printer setting and material composition to fit the laser system that was available in the laboratory. Briefly, overnight bacteria cultures were diluted 1:10 with gelatin (type A), Rose Bengal and LB broth solution and mixed for 10mins. This will yield a final concentration of 30% gelatin (type A) and 5-8.5mM of rose Bengal. A $\sim 40\text{-}\mu\text{L}$ droplet of the solution containing bacteria was spotted onto a glass slide and transferred to the 2PP system. Microstructures (circles or dumbbells) were then fabricated around the embedded bacteria (with an exposure time of .1ms and power 55-75mW). After fabrication, the un-cross-linked gelatin was washed out using multiple volumes ($\sim 0.5\text{ mL}$ per wash) of 37°C TSB. The sample was incubated at 37°C and observed at different timepoint with a confocal microscope.

5.3.8 Statistical analysis

Data are presented as the mean $\pm$ standard deviation and compared using two-way ANOVA test (Tukey's multiple comparisons test). All data were analysed using Prism software (version 6.01). $P < 0.05$ was considered to indicate a statistically significant difference.

5.3 RESULTS AND DISCUSSION

5.3.1 Proximal distal Microcolony assay

As mentioned, for urinary tract infection, the main causing agent is by *E. coli* strain UTI89. However, other microbes also play a role in causing the infection. Using the microcolony assay, different species were spotted 2cm apart and colony forming unit was counted after 24hrs and 48hrs(supplementary figure 3). Initial results shown that there was no change in growth for *E. coli* (>0.99), *S. aureus*(>0.99), and *P. aeruginosa*(>0.99) as shown in Figure 28a when they are grown individual as pairs or in triples. However, in the literature, it was shown that there was predatory effect when *P. aeruginosa* and *S. aureus* were grown together. *P. aeruginosa* either secretes antimicrobial products[198] or its siderophores[199] that competes for iron[200] and oxygen against *S. aureus*. At the same time, those experiments were done when both species were mixed together and it was also reported that in a wound model, *S. aureus* was found on the wound surface 30 μ m away from *P. aeruginosa* which is deeper in the wound. This result showed that spatial distance may play a role for multi-species bacteria interactions. With that, the microcolony assay was modified and the different species were mixed together instead of spotted separately. The killing effect reported in the literature was observed when *S. aureus* was grown with *P. aeruginosa* (<0.001). At the same time, there was a significant decrease in growth for *E. coli* when mixed with *P. aeruginosa* after 48 hours (<0.001 for both double and triple mix. It was reported that mutation in *E. coli* helped to maintain its growth when grown with *P. aeruginosa*[201]. That explained the slight decrease in growth at 24 hours although it was not that significant (0.3846 and 0.3765 for double and triple mix respectively), it maintained its CFU after 48 hours. Interestingly, *S. aureus* did not grow when mixed with *E. coli*(<0.001) . This was not observed when they were spotted separately as shown in Figure 28b. With these results, a deeper study was done to observe the mechanisms on how *E. coli* interacts with *S. aureus*.

Figure 28 a) CFU count of PAO1, UTI89 & MN8 on the Distal microcolony assay after 48hours.b) CFU count of PAO1, UTI89 & MN8 on the Mixed microcolony assay after 48hours. N =3 Black bar = Individual species, Red bar = PAO1 & MN8 grown together, Blue bar = PAO1 & UTI89, Yellow bar = UTI89 & MN8 and Green Bar = All 3 species grown together

5.3.2 Macrocolony assay analysis between *E. coli* and *S. aureus*

First, how fast the killing occurs between *E. coli* and *S. aureus* was determined. Based on the results (Figure 29), it seemed to have a significant decrease in *S. aureus* around 12 hours (<0.001) before it starts to completely wipe out by *E. coli* after 18hours. Thus, for further experiments, an 8 hours' time point and 24 hours' timepoint were looked at to see if there was a significant killing effect and a complete killing effect of *S. aureus* respectively.

Figure 29 CFU count of MN8 at different timepoints. Dark Grey bar = Individual UTI89, Light Grey bar = UTI89 enumeration with MN8, Black bar =Individual MN8 species, Red bar = MN8 enumeration with UTI89

The next question that existed was if *E. coli* strain UTI89 killing was specific to other *S. aureus* strains. Independent results of each *S. aureus* strain in Figure 30 showed that the killing pattern was seen throughout the different clinical strains, laboratory strain as well as methicillin-resistant *Staphylococcus aureus*(MRSA) strains with significant drops(<0.001) after 8 hours. Next, other *E. coli* strains were screened against *S. aureus*. Remarkably, M1655, which is a non-pathogen strain does not have the huge killing effect as compared to the UTI89 strain. One possibility is that UTI89 which contains two more siderophores than MG1655 to outcompete or kill *S. aureus* similar to *P. aeruginosa*.

CFU count of different Strains of *S.aureus* when grown with UTI89 over 24hours

Figure 30 CFU count of different strains of *S. aureus* grown with UTI89 at 8hrs and 24 hrs. Black colour = MN8, Blue bar = ISP427, Red bar = RN450, Green bar = HG001 & Orange bar = RN1

Figure 31 CFU count of UTI89, UTI89 Δ TonB and MG155 grown with MN8 at 8 hrs and 24 hrs.

Siderophores are iron chelating agents that bind to free iron and are taken by the cell. Siderophores are bacteria specific and have specific receptors to bring the iron into the cell. In order to bring the chelated iron into the cell, the TonB gene is required to create energy to open up the pores for the iron to enter. By using TonB mutant, a reduction in killing should be observed if the hypothesis is correct (Figure 31). However, from our results, there seemed to be a reduction in killing for the first 8 hrs but after 24 hrs, *S. aureus* was still killed by the mutant. Further confirmation was done with the single siderophore mutants and triple siderophore mutants (Supplementary Figure 3). It was observed that single siderophore mutants do not have the same effect but when all 3 siderophores were missing, the results were like the TonB mutant. It is possible that *E. coli* competition for iron play a minor role in killing as its only slows down the killing process.

5.3.3 Transposon screen and RNA sequencing results

In order to determine the true mechanism, a two-pronged approach was done. Transposon library and RNA seq. A UTI89 Transposon library was created where a kanamycin resistance gene is randomly inserted in the UTI89 genome, affecting the behaviour of the bacteria. By doing a growth curve assay of fluorescence *S. aureus* HG001-GFP strain with the different strains of *E. coli*, the intensity of the green fluorescence can be measured (Figure 32). For the initial testing, HG001 was grown with UTI89 and MG1655 and UTI89 Δ TonB to ensure there was a difference in intensity reading so that screening results can be differentiated. To subject any mutants from the transposon library for further screening, mutants should be similar to MG1655 results for further analysis. To perform the transposon screen, 15304 individual UTI89 colonies were picked to give an approximately 3X coverage to ensure that all the inserts were covered. From there, the primary screen gave about 120 hits and the secondary screen had 30 hits. These 30 hits were further analysed through multiple - step PCR or rescue cloning before using NCBI blast for comparative studies.

Figure 32 Fluorescence intensity reading of HG001 when grown individual (red), Mix with UTI89(beige), MG1655 (red with black). When UTI89 and MG1655 was taken for fluorescence there is nothing.

To investigate the mechanism governing *S. aureus*-mediated killing by *E. coli*, we surveyed the transcriptomes of both *E. coli* and *S. aureus* from the microcolonies assay using RNA sequencing (RNA-seq). We predicted modest differences in transcriptome of *E. coli* between single and mixed species since *E. coli* growth rate does not seem to change (Figure 31). In contrast, since *S. aureus* was reduced when mixed with *E. coli*, a difference should be observed in transcriptome.

Initial results for *E. coli* (Figure 33) showed that iron genes do not have any significant difference which correlate with the initial TonB and siderophore results. Other possible mechanism such as metabolism and Qs sensing also do not play a role. It is possible that other mechanisms such as antimicrobial could play a role since some of the hypothetical or uncharacterised proteins are not known yet. Once the transposon data have been finalised, RNA-seq data can be compared for the possible hits. For *S. aureus* (Figure 34), metabolism in terms of carbon, vitamins, nitrogen or even iron seem to be no different from the single species. This helped to confirm that metabolism or competition of nutrients by *E. coli* is not the method for killing *S. aureus*. Genes which had not been characterised yet seem to have the highest possibility for the true mechanisms.

Transcriptional gene expression profiling *UT189*/Mixed comparison

Figure 33 Transcriptional gene expression profiling of *E. coli* in microcolonies assay with *S. aureus*. Differentially regulated genes and functional categories (≥ 2 -fold change, FDR < 0.05)

Transcriptional gene expression profiling *HG001*/Mixed comparison

Figure 34 Transcriptional gene expression profiling of *S. aureus* in microcolony assay to *E. coli*. Differentially regulated genes and functional categories (≥ 2 -fold change, FDR < 0.05).

5.3.4 2PP of Gelatin microstructures

In this research, microscopic 3D printing strategies were implemented that enable multiple populations of bacteria to be organised in 3D geometries. Fabrication of gelatin biomaterials with 3D microstructures and sub-diffraction resolution has been successfully achieved due to focal cross linking of poly peptide molecules, through two-photon direct laser writing. It was determined that a 30% gelatin with 8mM rose Bengal hydrogel solution is sufficient for the printing of well-defined microstructures. If exposure time or power of the laser is increased, structures will burst, leaving bubbles within the structure. By using different OD of bacteria, cell density can be determined before mixing with the printing solution. Using this approach, the effect of dense cellular aggregates on each other can be observed, believed to play key roles in disease transmission. Fabricated scaffolds were biocompatible in nature as bacteria were still fluorescing, the presence of bacteria was observed from time $t=0$ to $t=8h$ (Figure 35). There seems to be no observable decrease in *S. aureus* when grown close to *E. coli* in the dumbbell structure (up to 50 micron apart). Micro fabrication of Bacteria species ought to follow the same trend but swelling phenomenon was observed after 8h. The bacterial species are biocompatible and in healthy condition but no interaction phenomenon was observed between them up to 8h. Figure 35c.

Figure 35 (a) Cylindrical Pattern containing *S. aureus* bacteria contained in Gelatin matrix (inset picture depicts the Confocal Image) (b) Cylindrical Pattern containing *E. coli* bacteria contained in Gelatin matrix (c) Interconnected Dumbbell pattern containing *S. aureus* and *E. coli* bacteria (inset picture depicts the Confocal Image)

Although proper structures of *E. coli* and *S. aureus* for observation can be obtained for up to 8 hours, more work have to be done before a constant structure for bacteria interactions studies can be established. Firstly, background noise from the uncured bacteria gelatin /rose Bengal solution affects reading of the confocal microscope. The number of washing steps have to be optimised as excess washing may destroy or wash away structures from the glass slide while

too little washing will result in a lot of background noise. Secondly, spotting of the solution have to be constant. This will affect the print settings resulting in varying structures. Due to the different spotting height created from the droplet, structures have to be printed with different exposure time and scanning speed. This affected the structure integrity and also the washing process. One possible method may be the use of spin coating to get an even coating or even the use of chamber glass slips to ensure equal amount of volume fills up the well. Finally, material properties of the gelatin with rose Bengal needs to be optimised and characterised to give the printed structures the right integrity to ensure long term growth and stability.

5.4 CONCLUSION

Fabrication of well-defined encapsulated bacteria gelatin scaffold structures was achieved by 2PP. Dumbbell structures containing both *E. coli* and *S. aureus* can be printed together. Initial confocal images showed that both bacteria species are growing well for up to 8 hours however no predatory interaction was observed when compared to the macro colonies due to the swelling phenomena. In the future, the matrix strength can be increased by functionalising the gelatin with methacrylate, in order to reduce the swelling phenomenon. Biocompatible polymers like PEGDA with different initiator can also be used as a potential candidate for the bacteria interaction studies, as the polymer matrix strength is high as compared to gelatin. The microcolonies assays showed that there is a spatial distance required for the predatory interaction of *E. coli* to take place. It is strain specific for the killing of *S. aureus* by *E. coli* UTI89 but not for *S. aureus* as all the strains tested during the research showed its susceptibility after 24 hours. Initial findings showed that the TonB gene or nutrient competition for iron may play a minor role in the killing *S. aureus*. Further optimisation of the PCR for transposon screen and additional repeats for RNA sequencing is required to conclude that *E. coli* has killed *S. aureus* as one biological sample was contaminated during the process. With a better understanding on how the bacteria interact and combining it with 3D printing, a better treatment for bacteria infection can be discovered and at the same time, provide a clearer picture on how the different spatial distances may affect bacteria interactions.

CHAPTER 6: CONCLUSION & FUTURE WORK

In this research, three different 3D printing techniques were used for constructing structures for cell studies. The first machine or technique was the use of a 3D bioprinter to create scaffolds that has the potential to be used in cardiac tissue engineering. Well-defined PCL and its CNT composites (up to 5% w/w) grid scaffold structures were fabricated with extrusion based technique. Slight enhancement in crystallinity when CNTs were added to the polymer matrix through DSC testing. Nanoindentation on the grid structures showed an increase in mechanical properties with increasing CNTs concentrations. The rate of biodegradation with *Pseudomonas* lipase revealed the structural and nanotube content dependence. MTT assay and fluorescence imaging results have shown that mouse cardiac myoblast cells were attaching to the scaffolds and remained in healthy condition after 4 days. It can be concluded that PCL-CNT nanocomposites with 1% CNT showed the optimal conductivity and stiffness for the proliferation of H9c2 cells. To study cell interactions with PCL and its nanocomposite matrix, polymer chain reaction and next generation sequencing can be done to determine how the cells behave in their environment. Further tuned degradation, cell compatibility and scaffold designs might be further utilised for the application of the scaffolds in tissue engineering for different cell types, as the PCL-CNT matrix can be enzymatically biodegraded after the formation of cardiac tissue.

Next, PEGDA hydrogel structures with different types of concentration of Irgacure 819 was printed with SLA system to act as a liquid chamber for studying cell-cell interactions. The honeycomb hydrogel structure made with the highest Irgacure 819 concentration of 1.0% swelled less, was stiffer but more brittle when compared to the other hydrogel with lower concentration of Irgacure 819. It was also noted that a higher concentration of Irgacure 819 resulted in a higher printability and resolution for the PEGDA hydrogel structures. Given that a hydrogel of high print resolution and accuracy was able to be printed, it showed the suitability of Asiga PICO2 in the fabrication of hydrogel scaffold. In order to have a proof of concept, a re-design in the scaffold might be needed. Given that the base of the scaffold had a thickness of 1mm, epifluorescence microscope might not be able to capture the fluorescence which should have occurred. PEGDA do not allow the adhesion of cells very well so structures could be printed directly onto the coverslip or glass slides to improve the attachment of cells. Another solution could be to sterilise coverslip before printing. Nanoparticles were incorporated in the

hydrogel to further enhance the mechanical properties and the printability as shown with the bioprinter research.

Finally, fabrication of well-defined encapsulated bacteria gelatin scaffold structures was achieved by 2PP for the study of *E. coli* and *S. aureus* interactions. Many bacteria live in their natural as polymicrobial biofilms. Initial mix and distal microcolonies showed that predatory effect was present after 8 hours when *E. coli* and *S. aureus* were mixed together. This indicated that there spatial distance is required for the predatory interaction of *E. coli* to take place. Further analysis also suggested s that the killing of *S. aureus* by *E. coli* UT189 is strain specific but not so for *S. aureus* as all the strains tested during this research showed its susceptibility after 24 hours. Initial findings showed that TonB gene is knocked out from the cell, nutrient competition for iron may play a minor role in the killing of *S. aureus*. With further optimisation of the PCR for transposon screen and additional repeats for RNA sequencing (one biological sample was contaminated), the mechanism on how *E. coli* kill *S. aureus*. *might be determined*. For spacing bacteria in the microscale, dumbbell structures containing both *E. coli* and *S. aureus* can be printed together using 2PP. 30% gelatin with 8Mm rose Bengal seems to produce optimal printing structure with the 2PP system. Initial confocal images showed that both bacteria species were growing well for up to 8hours, however, no predatory interaction was observed as compared to the macro colonies. Gelatin material is still being optimised as one main issue is the stability of it. Washing of printed substrate could be a reason why the structures collapse before any further experimentation can take place. With a better understanding on how bacteria interact and combining it with 3D printing, a better treatment for bacteria infection can be discovered and a clearer picture on how the different spatial distances may affect bacteria interactions.

To conclude, 3D printing machines have been around in the market and many different methods for printing are available, however, more time and systematic approaches have to be developed to achieve the possibility of printing of biomaterials. For my research, well-defined structures for both cardiac tissue engineering and microbiology have been shown. Although structures can be printed and they are compatible with biological materials, more research need to be done to ensure better overall stability so that longer experiments can be performed. As more interdisciplinary researchers are recruited into the field of 3D printing and together with the advancement in biomaterials, it is likely that 3D printing machines and techniques will be further improved over the years. This technology has the potential to not only change the way

that researchers approach collaboration but also our perceived limitations of experimental designs, particularly in biological studies where spatial control of samples or cells is critical for cell to cell interactions. With 3D printing, the search for the “killer application” can be achieved sooner.

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APPENDIX

Supplementary Table 1: Print settings done with PCL and its nanocomposites.

Materials	Feed rate (mm/s)	Pressure (bar)	Needle Size (diameter)	Temperature (°C)	Layers achieved
PCL	800, 1000, 1200	0.25	0.1	Room temperature	10
PCL-CNT-1	800, 1000, 1200	0.25	0.1	Room temperature	10
PCL-CNT-3	800, 1000, 1200	0.25	0.1	Room temperature	10
PCL-CNT-5	800, 1000, 1200	0.25	0.1	Room temperature	~7 to 9

Feed rate - Speed of the 3D printing stage.

Pressure - Amount of air used to force the material out of the syringe.

Needle size - The internal diameter of the needle tip.

Layer achieved - One layer refers to the combination of the horizontal and vertical strip.

Supplementary Figure 1: Stereo microscopic images of PCL and its nanocomposites at different feed rates (a) 800mm/sec. (b) 1000 mm/sec (c) 1200mm/sec. (Scale bar = 2mm)

Supplementary Figure 2: DSC thermograms of PCL and its indicated nanocomposites on its second run. Graph representing the change heat of fusion (J/g) and melting temperature w.r.t. CNT percentage in the sample.

Supplementary Table 2: Bacteria strains and description with their sources

Strain Name	Description	Source or Reference
<i>E. coli</i> UTI89	Uropathogenic clinical isolate	Chen et al., 2006
<i>E. coli</i> UTI89 Δ entB	In-frame deletion of entB	(Henderson et al., 2009)
<i>E. coli</i> UTI89 Δ iroA	In-frame deletion of iroA	(Lv et al., 2014)
<i>E. coli</i> UTI89 Δ ybtS	In-frame deletion of ybtS	(Henderson et al., 2009)
<i>E. coli</i> UTI89 Δ entB Δ ybtS	In-frame deletion of entB and ybtS	(Henderson et al., 2009)
<i>E. coli</i> UTI89 Δ TonB	In-frame deletion of TonB	Gift from Jeff Henderson, Washington University in St. Louis
<i>E. coli</i> MG1655	K-12 strain.	(Blattner et al., 1997)
<i>E. faecalis</i> OG1RF	Clinical isolate, ATCC47077	(Dunny et al., 1978)
<i>S. aureus</i> MN8		
<i>S. aureus</i> ISP147		
<i>S. aureus</i> RN1	NCTC8325	Novick, R. 1967.
<i>S. aureus</i> RN450		Novick, R. 1967.
<i>S. aureus</i> HG001	<i>rsbU</i> ⁻ laboratory strain	(Herbert et al. 2010)
<i>S. aureus</i> HG001-GFP	containing plasmid pCM29	(Pang et al 2010)

Supplementary Figure 3: Macrocolony Spotting of *P. aureginosa* (Top), *E. coli* (Bottom left) and *S. aureus* (Bottom Right) after 48hours. B) microcolonies count From left to right *P. aureginosa*, *E. coli* and *S. aureus*.

Supplementary Figure 3: CFU count of UTI89, and siderophore mutants grown with MN8 at 8 hrs & 24 hrs.