Directed cell alignment via extrusion-based 3D bioprinting for cardiac tissue engineering

Lee, Jia Min

2018

Lee, J. M. (2018). Directed cell alignment via extrusion-based 3D bioprinting for cardiac tissue engineering. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/75094>

<https://doi.org/10.32657/10356/75094>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**DIRECTED CELL ALIGNMENT VIA EXTRUSION-BASED
3D BIOPRINTING FOR CARDIAC TISSUE ENGINEERING**

LEE JIA MIN

School Of Mechanical & Aerospace Engineering

Nanyang Technological University

2018

**DIRECTED CELL ALIGNMENT VIA EXTRUSION-BASED 3D
BIOPRINTING FOR CARDIAC TISSUE ENGINEERING**

LEE JIA MIN

SCHOOL OF MECHANICAL AND AEROSPACE ENGINEERING

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

2018

ABSTRACT

Heart disease is the leading cause of death worldwide. Due to the limited regenerative ability of native myocardium, damaged myocardium cannot be restored. Advanced therapeutic approaches such as tissue engineering and regenerative medicine provides potential biological solutions to restore the function of damaged myocardium. Despite progresses in cardiac tissue engineering over the past decades, it is still far from mimicking the native myocardium. Some design considerations for engineering cardiac tissue includes inducing cell alignment, and attaining high cell density within a construct. Bioprinting, a computer-assisted technology, can potentially achieve the abovementioned considerations. In the native myocardium, fibre and cell orientation varies across different planar level. Bioprinting can potentially recapture this complexity through designing print path.

The purpose of this project is to fabricate an engineered cardiac tissue via bioprinting. Extrusion-based bioprinting was investigated in terms of printing parameters and material properties to achieve required print resolution for efficient nutrient exchange in bioprinted cell-hydrogel construct. Next, a design framework was developed to characterize and formulate materials for bioprinting. The design framework was applied for material formulation in extrusion printing of C2C12 cells.

Following which, a bioprinting strategy, termed support-assisted bioprinting, was established to produce three dimensionally defined constructs. Support-assisted bioprinting is a bioprinting strategy that uses a secondary material (support material) to provide mechanical stability for the primary material (build material) prior to crosslinking of the build material. Support-assisted bioprinting has demonstrated printing of three dimensionally defined cell-hydrogel, where hydrogel struts showed distinct 0°-90° angular difference at different height level. Cell alignment was achieved along the longitudinal axis of printed construct. The mechanism of cell-hydrogel remodelling process towards cell alignment in bioprinted construct was mapped for inducing cell alignment along strut orientation.

Lastly, enabling technologies in data and cell processing were developed as translational measures towards organ printing. In data processing, machine readable print path using G-code was generated to reflect the differences in fibre orientation of left ventricle wall. This shows the feasibility of fabricating bioprinted construct that better mimics the architecture of myocardium. In cell processing towards high throughput cell aggregates formation, the use of 3D printed microfluidics was studied.

These findings ascertained the potential of bioprinting towards directing cell alignment in a dimensionally defined construct.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude towards my supervisor Asst Prof Yeong Wai Yee for her constant support and guidance throughout the research. She has been an inspirational figure and has given me numerous opportunities to grow as a researcher.

I am also thankful to the technicians in Singapore Centre of 3D Printing (SC3DP) and Biological Lab. Specifically, I would like to thank Ms. Heng Chee Hoon for providing me with the necessary resources to carry out the project.

I would also like to extend my heartfelt gratitude towards fellow graduate students of SC3DP. The journey has been very enjoyable with you guys around.

Lastly, to my friends and family, your love and encouragement has kept me through this PhD road. No words can express my appreciation for your constant support throughout this wilful journey that I had chosen to embark upon. “Some people needs a lifetime for you to know them; while some people feels like you have known them for a lifetime”

TABLE OF CONTENTS

ABSTRACT.....	i
ACKNOWLEDGEMENT	iii
TABLE OF CONTENTS.....	iv
LIST OF FIGURES	xi
LIST OF TABLES	xxi
LIST OF SYMBOLS	xxii
LIST OF ABBREVIATIONS.....	xxiii
Chapter 1. Introduction.....	1
1.1. Background	1
1.2. Objectives.....	5
1.3. Scope.....	6
1.4. Thesis organization	7
Chapter 2. Literature review	9
2.1. Architecture of human myocardium	9

2.2. Cardiovascular diseases	13
2.3. Engineered cardiac tissue	16
2.3.1 Design requirements of engineered cardiac tissue.....	18
2.3.2 Fabrication techniques for engineered cardiac tissue	22
2.4. Formation of cell aggregates for tissue engineering	30
2.5. Directing cellular alignment in 3D tissue cultures	38
2.5.1 Stretch-induced alignment	42
2.5.2 Geometrical constraints	45
2.5.3 Current limitations	47
2.6. Bioprinting for cardiac tissue engineering	50
2.6.1 Bioprinting technologies.....	50
2.6.2 Biomaterials for bioprinting	59
2.6.3 Bioprinting strategies for 3D bioprinting of cell-hydrogel.....	61
2.6.4 Bioprinted cardiac construct	66
2.7. Research methodology	70

2.7.1 Influence of printing parameters and material property on print resolution of extrusion-based bioprinting.....	70
2.7.2 Material formulation for bioprinting of cell-hydrogel through extrusion	71
2.7.3 Support-assisted bioprinting for directing cell alignment	71
2.7.4 Enabling technologies for 3D cardiac organ printing.....	72
Chapter 3. Effect of printing parameters and material properties on print resolution of extrusion-based bioprinting	74
3.1. Introduction.....	74
3.2. Materials and methods	78
3.2.1 Hydrogel preparation	78
3.2.2 Rheological testing	78
3.2.3 Printing parameters.....	79
3.2.4 Mathematical model of fluid behaviour in extrusion-based bioprinting	80
3.3. Results and discussions	83

3.3.1 Effect of polymer concentration on viscosity and viscoelasticity of material	83
3.3.2 Effect of printing parameters on print resolution	87
3.3.3 Mathematical model relating process parameters with print resolution	91
3.4. Summary	98
Chapter 4. Material formulation and bioprinting of C2C12 cells.....	100
4.1. Introduction	100
4.2. Materials and methods	104
4.2.1 Synthesis of Gelatin Methacryloyl (GelMA)	104
4.2.2 Hydrogel preparation	104
4.2.3 Degradation studies	105
4.2.4 Encapsulated cell proliferation studies	105
4.2.5 Rheological test	106
4.2.6 Bioink formulation and printing parameters	106
4.2.7 Viability test of bioprinted cell	108

4.2.8 Microstructures of bioprinted hydrogel	108
4.3. Results and Discussions	110
4.3.1 Degradation studies	110
4.3.2 Cell proliferation studies	115
4.3.3 Rheological testing	117
4.3.4 Printing process	119
4.4. Summary	128
Chapter 5. Cell alignment with 3D organization through support-assisted bioprinting	129
5.1. Introduction	129
5.2. Materials and methods	133
5.2.1 Bioink formulation	133
5.2.2 Design of lattice construct	133
5.2.3 Tissue culture of the printed cell-hydrogel	134
5.2.4 Immunofluorescence microscopy	135

5.2.5 Quantification of cell alignment	135
5.2.6 Statistical analysis.....	136
5.3. Results and discussions	137
5.3.1 Characterization for support-assisted bioprinting.....	137
5.3.2 Cell alignment in bioprinted grid structure.....	146
5.4. Summary	168
Chapter 6. Enabling technologies for 3D bioprinting of the left ventricle wall	
170	
6.1. Data processing for freeform 3D bioprinting of the left ventricle wall	170
6.1.1. Introduction	170
6.1.2. Materials and methods.....	173
6.1.3. Results and discussions	176
6.1.4. Summary.....	183
6.2. 3D printed microfluidic platform for cell processing to form cell aggregates.....	184
6.2.1. Introduction	184

6.2.2. Materials and methods	187
6.2.3. Results and discussions	189
6.2.4 Summary.....	204
Chapter 7. Conclusions and recommendations for future work	206
7.1. Conclusion.....	206
7.1.1 Directed cell alignment using extrusion-based bioprinting	207
7.1.2 Bioprinting strategy for distinct 3D shape fidelity	209
7.1.3 Pre-printing technologies for processing data and cells	210
7.2. Future Work	211
7.2.1 Characterization of aligned cells	211
7.2.2 Improvement to print resolution model	212
7.2.3 Algorithms for generating print paths	213
7.3. List of publications.....	214
References.....	217

LIST OF FIGURES

Figure 1-1 Orientational difference of fibres as a) anisotropic direction according to the epicardium (presented in current disease models and engineered cardiac tissue) b) changing orientation of collagen fibres across different heart wall layer resembling native myocardium (inset of collagen fibre from [9])......	3
Figure 2-1 Cardiac morphogenesis showing the progression of tubular, looping, chamber formation and septation.	9
Figure 2-2 Organization level of myocardium. Functional units in myocytes begin with sacromeric proteins of sarcomere. Sacromeres are the basic units in myocytes. Myocytes fuse to form myofibrils. Endomysial fibre surrounds myofibrils. Perimysial fibre surrounds each muscle bundle.	10
Figure 2-3 Schematic representation showing the fibre orientation and volume composition at different heart layer with different percentage composition of heart layer.....	12
Figure 2-4 Changes in fibre orientation of left ventricle mapped across different plane and at different position. Graph adapted from values obtained by Rohmer <i>et al.</i> [19].	13
Figure 2-5 Conventional and Advanced therapy for Myocardial Infarction.	15

Figure 2-6 Design factors for engineered cardiac tissue guided by mechanical and biochemical properties of cardiac tissue.	17
Figure 2-7 Design considerations in engineering cardiac tissue.....	19
Figure 2-8 Spheroid formation techniques [A-D conventional spheroid formation techniques; E-G microfluidic-based spheroid formation techniques] a) hanging drop method, b) concave micromould, c) spinning culture flask, d) rotary cell culture, e) droplet formation of multiphase flow, f) array of microwells, and g) rotational flow in microchamber.....	31
Figure 2-9 Mechanical cues that influence cell alignment classified broadly into stretch-induced alignment versus geometric constraints.	41
Figure 2-10 Classification of bioprinting technology Computer-aided bioprinting allows spatial control on the deposition of bioactive materials.	51
Figure 2-11 Schematic representation of the different jetting technology for depositing cells, biomaterials and biomolecules.....	53
Figure 2-12 Differences between (left) laser guided direct writing (right) laser induced forward transfer.	55
Figure 2-13 Schematic representation of stereolithography (left) and dynamic optical projection (right).	56

Figure 2-14 A bioprinting process that uses combination of different printing strategies to improve print fidelity and resolution.	61
Figure 2-15 Research methodology that investigate on the bioprinting process in i) controlling print resolution and definition ii) material formulation.	73
Figure 3-1 Resultant forces acting on a finite volume within a cylindrical geometry.	81
Figure 3-2 Rheological data of alginate hydrogel (A) Viscosity profile of alginate hydrogel with varying concentration (B) Amplitude sweep of alginate with different concentrations.	84
Figure 3-3 Graph showing the non-linear relation between moving stage speed with dimension of construct width of different alginate composition A) 12:1 B) 12:2 C) 12:3.	88
Figure 3-4 Controlling pore size of grid printing (white scale bar: 500 μ m).....	89
Figure 3-5 Percentage change of hydrogel width on different substrate.	90
Figure 3-6 Cross-sectional area of hydrogel struts printed A-C) ideal extrusion based bioprinting toolpath with circular cross-sectional area D-F) extrusion based bioprinting toolpath with elliptical cross-sectional area G) mathematical equation describing the different cross-sectional area.	91

Figure 3-7 A Preliminary model set up to describe volume flow rate of hydrogel extruded.....	93
Figure 3-8 Preliminary model relating width of hydrogel struts to flow rate of hydrogel extruded and moving speed of platform.	94
Figure 4-1 Framework in material selection process for bioprinting.	103
Figure 4-2 Mass change rate of different hydrogel compositions immersed in 2.5U/mL Collagenase Type II to investigate on the effect of polymer content in influencing degradation rate.	112
Figure 4-3 Cell proliferation study using PrestoBlue®.	116
Figure 4-4 Rheological properties of 10% GelMA composite samples A) Frequency sweep B) Complex viscosity.....	118
Figure 4-5 Fluorescence microscopy comparing extrusion of bioink with varying concentration for a time frame of 15 s across different pressure settings (white scale bar = 200 μ m; green and red denotes Live and Dead cells respectively). Higher cell death was observed in 15M2A10C as indicated by region bounded in dotted red.	120
Figure 4-6 Graph comparison between cell viability between different bioink composition containing different loading cell density.....	122

Figure 4-7 (A&B) Fluorescence microscopy of LIVE (green) and DEAD (red) cells respectively. (C&D) Data acquisition to quantify number of cells using ImageJ for LIVE and DEAD cells respectively. (E) Comparison between cell viability (* denotes significant difference at $\alpha=0.05$, ** at $\alpha=0.10$ using one tail t-test). 123

Figure 4-8 Comparison between cell viability of cells exposed to UV and as printed (NUV). 125

Figure 4-9 Schematic illustration of SEM sample preparation showing microstructures in the exposed region (A); (B&C) Lamination effect of printed hydrogel (red scale bar: 20 μm ; white scale bar: 200 μm). 126

Figure 4-10 SEM images showing microstructure of casted hydrogel A) 5 mg/mL Collagen B) GelMA with Alginate (10M2A) and (C&D) bioprinted 10M2A showing directional preference along different location (red scale bar: 20 μm ; blue dotted line in C & D denotes the side at which hydrogel is printed unto substrate of glass slide). 127

Figure 5-1 Schematic illustration to demonstrate the lack of shape fidelity demonstrated in a 3-layered grid construct using bioprinting. Microscopic images taken from [254]. 130

Figure 5-2 Schematics of using support-assisted bioprinting process in using support material (Pluronic F127) with build material (bioink: GelMA and

Alginate with cells). Pluronic F127 provides temporary support prior to UV curing of the bioink for enhanced stability in cell culturing.	134
Figure 5-3 Cross sectional profile of support material (Pluronic F127) deposited on a glass slide with varying applied pressure. Support material was printed via a 21G needle, at constant printing speed of 600 mm/min. Distance between support struts were kept at 1mm.	138
Figure 5-4 Schematic diagram to illustrate support-assisted bioprinting of build material (red) between support material (grey).	139
Figure 5-5 Comparison between the surface profile of 0°-90° struts printed with support-assisted bioprinting and direct bioprinting of build material (without support). Confocal microscopy of samples taken using Keyence VK-X210. Distinct differences between first and second layer is observed in support-assisted bioprinting; whereas material accumulation (circled in pink) is observed in direct bioprinting.....	141
Figure 5-6 Comparison between direct bioprinting without support and support-assisted bioprinting on the struts dimension (*asterisks shows the statistical significance between groups at $\alpha=0.05$). Direct bioprinting without support produces thicker hydrogel strut width with higher deviation; whereas support-assisted bioprinting produces finer resolution and lower deviation with distinct z-layer differences.....	143

Figure 5-7 Comparison between cells seeded on scaffolds versus cells printed within the hydrogel. (red scale bar: 200 μm).	146
Figure 5-8 Growing of cell cluster observed in Day 2 of culture. Red dotted circle indicates area of clustered cells due to inhomogeneous cell seeding in Day 0.	147
Figure 5-9 Cell alignment profile of seeded cells at day 4. Blue dotted regions identified the change in cell alignment at varying z plane. (red scale bar: 100 μm).	148
Figure 5-10 Timepoint study of cell alignment in bioprinted cell-hydrogel struts across 14 days. Red arrows and regions indicate the elongation of cells.	151
Figure 5-11 Confocal fluorescence microscopic images showing F-actin (green) and DAPI (blue) of the printed hydrogel struts (white scale bar = 50 μm).	153
Figure 5-12 Cell alignment profile at the perimeter (Area A).	154
Figure 5-13 Cell alignment profile at the turning angle (Area B).	156
Figure 5-14 Cell alignment profile at the interstrut (Area C).	157
Figure 5-15 Cell alignment analysis over short range of 1 mm (scale bar: 100 μm).	159

Figure 5-16 Cell orientation over the interface between vertical and horizontal struts. Brightfield microscope image of cell alignment viewing from bottom layer of 0° struts, upper layer of 90° struts and the transitioning phase between 0°-90° struts. Angle of cell alignment is quantified and mapped along various position of the struts.....	160
Figure 5-17 Brightfield and fluorescence microscopy images to demonstrate the printing of cells varied at different angle orientation (Green: F-actin; Blue: DAPI; Red scale bar: 200 μm).	161
Figure 5-18 Cell spreading observed at peripheral of strut during day 4 of culture indicated by red arrows.	165
Figure 5-19 Mechanism of cell alignment in bioprinted strut.	167
Figure 6-1 Process flow for file preparation for bioprinting, comparing between the two different file processing methods.	173
Figure 6-2 CAD models for printing of free-standing coil and left ventricle wall.	174
Figure 6-3 Schematic illustration of print process using conventional layer-by-layer printing versus file segmentation for bioprinting of free-standing coil. Free-standing coil was used as the reference model for development of file preparation framework to print left ventricle wall.	175

Figure 6-4 Schematic illustration of print process using conventional layer-by-layer printing versus file segmentation for bioprinting.....	178
Figure 6-5 3D model and image of the left ventricle with build (red) and support (green) material. Curvature of printed left ventricle preserved after support material removal (curvature mapped with red dotted line).....	179
Figure 6-6 Schematics for generation of print path with different angular orientation mimicking changes in the transmural left ventricular wall. Fibre orientation of native myocardium mapped out in [18, 19, 21, 22].	181
Figure 6-7 SEM images of printed channels. Polyjet printed channel at A) 90° B) 30°; FDM printed channel at C) 90° D) 30°	190
Figure 6-8 Ra of the parts printed for various wall angles.....	191
Figure 6-9 SEM images of other channels. Round channels printed by A) Polyjet B) FDM; C) Slanting channel by polyjet yielded rougher wall.....	192
Figure 6-10 Water contact angles of the model materials.	194
Figure 6-11 Cell viability under various treatment conditions.	196
Figure 6-12 Fluorescence staining of cells on FullCure 720 subjecting to 70% ethanol and UV after overnight incubation.....	197

Figure 6-13 Direct printing of rotational flow device. A) Channel blocked by support materials. B) Channel distorted by material swelling.	199
Figure 6-14 Assembly of the rotational flow device.	199
Figure 6-15 Printing of layered rotational flow device. A) Channel blocked by adhesive during bonding. B) Disturbed flow due to ineffective bonding.	200
Figure 6-16 Assembly of the gradient generator (Left) CAD design of gradient generator (Right) Printed gradient generator.	201
Figure 6-17 Close up view of the gradient generator. Left) Before removal of support material. Right) After removal of support material.	202

LIST OF TABLES

Table 2-1 Comparison of different fabrication techniques in engineered cardiac tissue	27
Table 2-2 Cell-biomaterial manipulation using microfluidic design for tissue engineering applications	37
Table 2-3 Comparison of different bioprinting techniques.....	57
Table 2-4 Summary of different 3D bioprinting strategies.....	65
Table 2-5 Bioprinted cardiovascular and cardiac-related tissue	68
Table 3-1 Comparison of different extrusion systems	77
Table 3-2 Comparison between two extrusion-based printing strategies for improved printability	86
Table 4-1 Different material compositions for evaluation on biological and mechanical properties	105
Table 4-2 Variation in material composition and printing parameters for cell viability testing.....	107
Table 6-1 Consideration factors for file segmentation	177
Table 6-2 Sterilization condition of 3D printed material.....	188

LIST OF SYMBOLS

F_Z	---	net force acting on fluid element
τ_r	---	shear stress
V	---	fluid's velocity
$\dot{\gamma}$	---	shear rate
$\dot{\gamma}_o$	---	reference shear rate
V_o	---	velocity profile of fluid
R	---	inner radius of needle
η_o	---	initial viscosity
n	---	power law index
Q	---	volumetric flow rate
Q_m	---	mass flow rate
ρ	---	density of fluid
V_m	---	speed of moving stage

LIST OF ABBREVIATIONS

AM	Additive Manufacturing
CaCl ₂	Calcium Chloride
CAD	Computer-Aided Design
CFs	Cardiac Fibroblasts
CMs	Cardiac Muscle Cells
CT	Computerized Tomography
DA	Degree of Methacrylation
dECM	Decellularized Extracellular Matrix
DLP	Digital Light Processing
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DS	Degree of Substitution
DT-MRI	Diffusion Tensor Magnetic Resonance Imaging
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
FDM	Fused Deposition Modelling
FFT	Fast Fourier Transform
GelMA	Gelatin Methacryloyl
HA	Hyaluronic Acid
HAMA	Hyaluronic Acid Methacryloyl

HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
hMSCs	Human Mesenchymal Stem Cells
HUVECs	Human Umbilical Vein Endothelial Cells
ID	Inner Diameter
LGDW	Laser Guided Direct Writing
LIFT	Laser-Induced Forward Transfer
LV	Left Ventricle
MMP	Matrix Metalloproteinase
NaOH	Sodium Hydroxide
NC	Numerical Control
OD	Outer Diameter
PBS	Phosphate Buffer Saline
PCL	Polycaprolactone
PDMS	Polydimethylsiloxane
PEGDA	Poly(ethylene glycol) Diacrylate
PF	Poly(ethylene glycol)-Fibrinogen
PLGA	Poly(lactic-co-glycolic acid)
PLLA	Poly-L-lactic Acid
PMGI	Polymethylglutarimide
PMMA	Poly(methyl methacrylate)
PNIPAm	Poly(N-isopropylacrylamide)
RGD	Arginylglycylaspartic Acid

SEM	Scanning Electron Microscopy
SLA	Stereolithography
SLS	Selective Laser Sintering
UV	Ultra-Violet
VPP	Vat Photopolymerization

Chapter 1.Introduction

1.1. Background

Cardiovascular disease, one of the world's leading cause of death, possess a relatively large market in the field of regenerative medicine. In the past decade, medical advances have decreased mortality rates related with cardiovascular diseases such as acute coronary syndromes, valvular and congenital heart disease, uncontrolled hypertension and arrhythmias [1]. However, death rate from heart failure remains high with 50% of heart failure patient dying within 5 years of diagnosis [2]. One possible reason attributing to the stagnated improvement of heart failure's mortality could be explained by repeated episodes of cardiovascular diseases despite previous intervention. This suggests a plausible case where risk of heart failure in patients has been reduced but the root of problem has not been solved [1].

Damaged myocardium cannot be restored on its own as native cardiomyocytes have limited regenerative abilities. Hence, cardiac tissue engineering aims to provide biological solutions to restore the ill functioning heart and has the largest potential in regenerative medicine [3]. Researchers aim to engineer heart tissue as potential solution for corrective, reparative, and restoration of ventricle function. The motivation for cardiac tissue engineering is to provide a tailored microenvironment for culturing of myocytes. Through the combination of

biomaterials and non-myocyte cells, the fabrication of myocardium tissue is optimized to produce aligned, heterogeneous engineered tissue with high cell density [4-8].

The current fabrication of cardiovascular tissue models or engineered cardiovascular tissue is in accordance to the heart's surface, the epicardium (Figure 1-1a). Engineered cardiac tissues have been fabricated with cells unidirectionally alignment to create engineered cardiac tissue with anisotropic properties. However, the cellular and fibrillar organization of the heart differs across the ventricular wall (Figure 1-1b). The need for fabricating directionally defined and high cell density construct is the basic requirement of an engineered cardiovascular tissue. To date, there is no existing fabrication strategy that can demonstrate directionally controlled cell alignment that resembles the torsional organization of the native myocardium. In this research, engineered cardiovascular tissue will be fabricated taking considerations in the changing orientation of fibre and cells in the transmural direction resembling the different layers of heart wall.

Figure 1-1 Orientational difference of fibres as a) anisotropic direction according to the epicardium (presented in current disease models and engineered cardiac tissue) b) changing orientation of collagen fibres across different heart wall layer resembling native myocardium (inset of collagen fibre from [9]).

Bioprinting, one of the nascent fields in tissue engineering, arises as a potential fabrication method to produce anatomically accurate and functional engineered tissue [10]. With the knowledge of cell-material interaction from conventional fabrication of engineered heart tissue, bioprinting adopts the design-dependent approach to deposit biomaterials and cells with computerized control. Specifically, extrusion-based bioprinting has been widely used due to the wide range of processable materials and ease of operation. Moreover, printing large amount of cells with high density is favourable using extrusion-based bioprinting.

Bioprinters will be used as a tool for biofabricating dimensional, geometrical and cellular defined constructs. Bioprinting, a computer-assisted technology, allows depositing cells and biomaterials (soft hydrogels) onto predefined coordinate position. Moreover, using different printing cartridges, different cells can be

deposited and co-cultured. Although several studies have incorporated the use of bioprinting for cardiovascular tissue engineering, there are limited reports on defining directional cell alignment that can mimic the fibre orientation of native myocardium. As such, the use of bioprinting in directing cell alignment aims to recapture the angular differences in fibre and cell alignment transmurally of the left ventricular wall. This thesis shows the viability of using support-assisted bioprinting in producing dimensionally defined construct through the development of a printing strategy for directing cell alignment.

1.2. Objectives

The main objective of this research is to obtain directed cell alignment using extrusion-based bioprinting. The project aims to accomplish the following:

1. To analyse the process of extrusion-based bioprinting. The relationship between printing parameters and material properties with print resolution will be modelled and validated with physical experiments.
2. To devise a framework in selecting material suitable for extrusion bioprinting. The material composition is selected such that the bioprinted construct is structurally stable for cell culturing while it is degradable to encourage cell adhesion, proliferation and attachment.
3. To establish print definition such that cell-laden hydrogel is deposited with spatial control.
4. To obtain cell alignment in bioprinted tissue constructs. With the print definition established, cell alignment at different planar level is essential to recapture the microstructure differences in the native myocardium.
5. To develop enabling technologies that would assist pre-printing processes. Data processing of print files is essential to determine the toolpath for cell-laden hydrogel deposition. Pioneering the use of x, y, z coordinate-based deposition will allow printing of engineered constructs resembling the anatomical form of tissue. Designing novel microfluidic platform for cell aggregates formation will increase cell density of printed tissue.

1.3. Scope

The scope of this research includes:

- i) Printing parameters and material properties will be investigated, and a model will be established for controlling print resolution and shape fidelity of bioprinted constructs.
- ii) Materials will be formulated for extrusion-based bioprinting of C2C12 cells. A material formulation framework will be designed for material selection.
- iii) A bioprinting strategy will be proposed to enhance shape fidelity in bioprinted constructs. The printing strategy coupled with the effect of extrusion-based bioprinting will be used for directing cell alignment.
- iv) Enabling technologies for data and cell processing in 3D bioprinting will be carried out as proof-of-concept towards bioprinting of the left ventricular wall.

1.4. Thesis organization

Chapter 1 introduces the background of research on cardiac tissue engineering, bioprinting and the motivations for this research. The objectives and scope are also listed.

Chapter 2 reviews previous efforts in cardiac tissue engineering. Additionally, design considerations in engineering cardiac tissue are highlighted. State-of-the-art bioprinting technologies are presented as fabrication technique for cardiac tissue engineering.

Chapter 3 studies the relationship between printing parameters and material properties with printing resolution. A mathematical model describing fluid behaviour is established to describe the correlation.

Chapter 4 evaluates on the different composition of gelatin methacryloyl (GelMA) for bioprinting. Evaluation of material formulation is assessed based on cell-based and process-specific requirements.

Chapter 5 investigates on support-assisted bioprinting as the printing strategy for controlling print definitions and shape fidelity. The strategy is used to print 0^0 - 90^0 cell-laden lattice structure. Cellular alignment is achieved at different planar layer of the structure.

Chapter 6 identifies enabling technologies that aids data and cell processing for bioprinting in the pre-printing phase. A framework for data file processing is designed for controlling print path that resembles the fibre orientation of native myocardium. Also, 3D printed microfluidic platform is characterized and evaluated for application towards generation of cell aggregates.

Chapter 7 concludes this research with recommendations for future work.

Chapter 2. Literature review

2.1. Architecture of human myocardium

The human heart comprises of approximately 2 billion cells [11, 12] with intricate myocardium architecture comprising of non-cardiomyocytes cells and fibrillary structure. To understand the myocardium architecture, it is essential to know the development of human heart. Cardiac development, or cardiac morphogenesis, comprises of mainly four stages, tubing, looping, chamber formation and septation [13] (Figure 2-1). As such, the ventricle architecture can be described either as “ventricular muscle band” [14] or a “continuum entity” [15].

Figure 2-1 Cardiac morphogenesis showing the progression of tubular, looping, chamber formation and septation.

Despite the differences in describing the myocardium architecture, the myocardial strands reveal general longitudinal orientation of myocyte arrangement [16]. To appreciate the helical fibre orientation in myocardium, it is

first essential to understand the different organization levels of myocardium. The heart comprises of four distinct layers ranging from pericardium (outer), epicardium, myocardium, and endocardium (inner). Each distinct layer comprises of myocardial laminar sheets with muscular fibres of three to four cell layer thick (Figure 2-2) [17]. These laminar layers, also known as sheetlets, orientates and contributes to wall thickening during systolic shortening [18].

Myocytes are organized in bundles of myofibres within cardiac microstructure. The orientation of myofibres changes from a right-handed to a left spiral-like arrangement. Cylindrical myocytes are in diameters of 20 – 30 μm with length up to 150 μm [16]. Within each muscle fibre, units of sacromeres make up the basic unit of muscle fibre. Sacromeral proteins are the smallest unit in the myocardium which contributes to the contractile function of myocardium.

Figure 2-2 Organization level of myocardium. Functional units in myocytes begin with sacromeric proteins of sarcomere. Sacromeres are the basic units in myocytes. Myocytes fuse to form myofibrils. Endomysial fibre surrounds myofibrils. Perimysial fibre surrounds each muscle bundle.

The native myocardium is composed of 3 main collagen fibres namely i) epimysial ii) perimysial iii) and endomysial. Epimysial fibres have large diameters in the range of several micrometres and provide the external structure that surrounds myocardium; Perimysial fibres ranges in the submicron level which runs parallel with the long axis of muscles and are responsible for cell bundles contraction; Endomysial fibres ranges in 20 – 100 nm and wrap around individual cardiomyocytes.

Fibre orientation and wall thickness vary from epicardium to endocardium. These gradual changes result in torsional motion of the heart which is relative to cardiac's ejection fraction [16, 19, 20]. Using confocal microscopy, Pope and co-workers identified extensive meshes of collagen fibres with varying degree of orientation across the walls of rat's left ventricle [9]. Advanced imaging techniques such as diffusion tensor magnetic resonance imaging (DT-MRI) have been used to map the cardiac fibre and laminar architecture [18, 19, 21, 22] (Figure 2-3).

Figure 2-3 Schematic representation showing the fibre orientation and volume composition at different heart layer with different percentage composition of heart layer.

Changes in the angle of the fibres are mapped and reconstructed from Rohmer *et al.* (Figure 2-4) [19]. The fibre angular difference varies from the position and plane of visualization (basal or apical; anterior, lateral, posterior and septal). For instance, fibre orientation in the apical region varies across anterior to septum plane from -22.91° to -47.9° at the epicardial layer 53.42° to 29.73° at the endocardial layer.

Figure 2-4 Changes in fibre orientation of left ventricle mapped across different plane and at different position. Graph adapted from values obtained by Rohmer *et al.* [19].

2.2. Cardiovascular diseases

While myocardial infarction alone does not lead to heart failure, problems associated with cell death arising from decrease in blood supply will contribute partly towards heart failure. In general, myocardial infarction occurs when there is occlusion/reduction in the amount of blood supplies to the heart. Cardiomyocytes undergo apoptosis due to the decline in the amount of blood/nutrients. Apoptosed cells are removed by macrophages through secreting enzymes that degrade the dead cells.

In early remodelling stage, migration of macrophages, monocytes and neutrophils into infarct zone degrade intermyocyte collagen struts. This results in wall thinning and ventricular dilation, causing an increase in wall stresses. Later

stage of remodelling involves myocyte hypertrophy and alteration of ventricular architecture to evenly distribute wall stresses. This causes thinning of the heart wall [23]. Hence the term, dilated cardiomyopathy.

Solutions for Failing Heart

The golden solution to failing heart is organ transplantation. However, there is shortage of available organs for transplantation [24-26]. Alternatively, medical interventions such as pharmacological therapy, corrective surgeries and medical devices have been implemented. Surgical intervention for myocardial infarction aims at resupplying blood flow into infarcted area via coronary bypass graft. On the other hand, corrective surgery restores left ventricle's elliptical shape to enhance ejection fraction. These conventional approaches lack regenerative ability, hence the need for biological solutions for advanced and bioinspired approaches. Figure 2-5 shows some conventional and advanced therapy for myocardial infarction.

Figure 2-5 Conventional and Advanced therapy for Myocardial Infarction.

A potential biological solution is cell therapy where cells are injected directly at infarct site or intracoronary to repair and regenerate myocardium [27-30]. Clinical trials have been performed to show safety and feasibility of using cell-based therapy. It has shown that these cell-based therapy aids in increasing mass of viable myocardium while promoting angiogenesis through paracrine effects [28, 31]. However, injecting cells directly suffers from low cell survivability and low cell retention at injured site.

Alternatively, biomaterials, used as vehicles for cell delivery, have shown to increase in cell retention at injection site. Hydrogels, which are common in cell injection, help protect cells from shearing during injection while increasing cell

retention [31, 32]. Moreover, injected biomaterials alone reduce ventricular remodelling through Laplace's Law.

Another form of cell therapy for cardiovascular disease uses cell culturing methods to mature cells prior to implantation forming cardiac patches. The effect of three dimensionality culture for cells has been demonstrated by Zhang *et al.* where longer sarcomeres are formed with higher electrical conduction velocity [33]. While cardiac patches can potentially be used as a cell-based therapy for cardiovascular diseases, an immediate application for engineered heart tissue will be as disease models and drug testing purpose [34, 35]. Davis and co-workers have demonstrated such drug testing model for in vitro evaluation of cardiac sodium channel disease using human induced pluripotent stem cells [36].

Nonetheless, extensive research has been done on various fabrication methods for cardiac patches (or engineered cardiac tissue). Mainly four forms of engineered heart tissue are presented i) hydrogel with cardiac cells ii) cell sheet layers iii) cellularization of prefabricated polymeric matrices iv) repopulating decellularized tissue (Figure 2-5). One of the emerging fabrication methods in tissue engineering has been bioprinting, a computer assisted technology.

2.3. Engineered cardiac tissue

The role of biomaterials used in scaffold based fabrication of myocardial tissue is to protect cells from aggressive environment, temporal support for cell

engraftment and retention, while providing biochemical and biophysical cues for cell survival and angiogenesis.

This section describes the technologies used for fabricating engineered cardiac tissue and can be broadly divided into two categories, namely scaffold and scaffold-free fabrication. Ideally, engineered cardiac tissue should have similar mechanical properties (Modulus: 0.2 – 0.5 MPa at end diastole; Tensile Strength: 3 – 15 kPa; Contractile force: 2 – 4 mN/mm²) and electrical propagation velocity: 25 cm/s) as human myocardium [37, 38] (Figure 2-6) [35].

Figure 2-6 Design factors for engineered cardiac tissue guided by mechanical and biochemical properties of cardiac tissue.

2.3.1 Design requirements of engineered cardiac tissue

Several factors are considered when engineering a cardiac tissue. Firstly, the native heart comprises of heterogeneous population of cells ranging from endothelial cells to cardiomyocytes to fibroblast. It was also hypothesized that cardiomyocytes present at different transmural section play different functional roles. Secondly, the architectural features of native heart differ at different hierarchical level. At milliscale level, matrix anisotropy induces aligned myofibres while having varying spatial arrangement across transmural direction; In the micrometre scale, high cell density of native myocardium requires support from vascularization [39]. Moreover, with the huge loss of cardiomyocytes during infarction, engineered cardiac tissue aims to provide an *in vivo* solution to cardiovascular diseases. Lastly, maturation of engineered cardiac tissue is required to yield a structure that restore and repair functional loss after cardiovascular events. Figure 2-7 summarises the key design considerations in engineering cardiac tissue.

Figure 2-7 Design considerations in engineering cardiac tissue.

2.3.1.1 Heterogeneous cell population

Cardiomyocytes ironically forms the minority of cell population in heart yet giving rise to the bulk volume of the heart, whereas endothelial cells dominates the non-myocytes population [40]. Fibroblast dominates the nonmyocyte population and is in direct contact with cardiomyocytes [41]. Other nonmyocytes population includes endothelial cells, adipocytes and neurons.

Myocytes-nonmyocytes coupling was demonstrated by Camelliti and co-worker postulating the contribution of nonmyocytes to cardiac electrophysiology [42].

In situ imaging of functional gap junctions between cardiac fibroblast and cardiomyocytes shows propagation of electrical signal across sinoatrial tissue.

On the other hand, endothelial cells are able to produce survival and trophic

signals to cardiomyocytes [43]. In addition, angiogenesis was enhanced in cardiac cell sheets cocultured with endothelial cells [44].

Triculture tissue constructs (cardiomyocytes, endothelial cells, and fibroblast) has been used for most engineered cardiovascular tissue to enhance graft vascularization [4, 5]. The interplay between cardiomyocytes and non-myocytes is complex yet the synergy is vital for engineering vascularized cardiac tissue. Another approach in triculture of myocytes and non-myocytes cells follows a sequential pathway of first seeding both endothelial and fibroblast cells subsequently with cardiomyocytes. Iyer *et al.* demonstrated that simultaneous seeding of triculture population of cells produce non-contractile organoids with absence of Connexin 43 stains [45].

2.3.1.2 High cell density

With the huge number of cell loss during myocardial infarction, many of the engineered cardiac tissue aim at fabricating high cell density construct. Moreover, microscopy has revealed highly dense composition in native cardiac tissue. High cell density refers to the higher cell-cell connection within the construct [46].

Close proximity between cells renders effective cell-cell junction for electrical propagation and conductivity. Hence, one of the significant indicators of having a functional engineered heart tissue is to identify gap junction protein Connexin 43. Interestingly, synchronous beating is demonstrated with cardiac cell cluster

while synchronous contraction between each cell cluster is lacking [47]. The lower expression of Connexin 43 between cell clusters explains the asynchronous beating. This signifies a need to produce engineered heart tissue with cell closely connected.

Another factor that arises with concern for high cell density construct is the need for vasculature network. Cardiac tissue has high metabolic demand for oxygen (27.6 noml/mg [protein.min] [48]. Hence, vasculature network has been incorporated in engineered cardiac tissue to achieve cardiac tissue of considerable thickness [49, 50].

2.3.1.3 Fibre alignment for torsional motion

Fibre orientation in myocardium ranges from left handed helices at epicardium to right handed helices at endocardium. Fibre orientation, degree of contraction and relaxation of myocardium, balance between contraction of subendocardium and subepicardium are three factors that contributes to the rotational motion of heart [51]. In infarcted myocardium, changes in helix angle of fibre tracts are observed across all layers of myocardium with subepicardium having the greatest positive shift [52]. Functional engineered myocardium patch should also restore normal 3D myofibres architecture while generating functional cardiomyocytes.

2.3.1.4 Cell alignment in cardiac architecture

In native ventricular myocardium, cardiomyocytes are elongated and aligned along the fibre direction. The contractile motion of heart is defined by cardiac cells alignment. Aligned and elongated cardiomyocytes is vital in excitation-contraction coupling of heart via efficient intracellular handling of calcium [6-8]. Cell shape influences cardiac function through affecting downstream regulatory roles such as extracellular matrix coupling, gene expression, impulse propagation, excitability and contraction [53].

2.3.2 Fabrication techniques for engineered cardiac tissue

2.3.2.1 Cell sheet technology

Cell sheet technology involves cell culturing on modified thermo-responsive polymer surface, Poly(N-isopropylacrylamide) (PNIPAm). With temperature change, the cells that are grown to full confluency on the coated culture plate is dislodged as an entire layer with cell-cell junction intact. This method fabricates cell sheet and has provided reconstructive applications towards cornea, periodontal, bladder, liver, trachea, renal, oesophageal, and cardiac [54]. Cell sheet technology proves to be a better alternative as compared to conventional cell injection therapy as it solves limitations previously presented in cell injection, such as cell retention on site. With this technology, Shimizu and co-worker have developed novel methodology to construct layered cardiomyocytes sheets with

established electrical communication between cell layers without the use of scaffolds [55].

In a study by Miki, cardiac cell sheet made of induced Pluripotent Stem Cells was implanted directly over the scar tissue without suture [56]. The retention of cells in the injured site is higher since cells are transplanted via planar layers of cell sheet as compared to individual cell suspension during cell injection. Moreover, cell sheet is transplanted into host tissue without sutures, representing a potential for minimally invasive treatment. Other than cell survivability during transplantation, another concern with cell sheet harvest is the lack of vascularization. Hence, bioreactors and microchannel perfusion procedures are used to vascularize thick layer of cell sheet [49].

One limitation of such technology is the limited cell number generated from each cardiac cell sheet. Based on mathematical calculation, cell sheet seeded in a 10 mm well plate used by Miki *et al.* [56], yields approximately 9.5×10^4 cells (from Corning 48 Well Plate of 11 mm diameter). These values are significantly less than 8×10^6 cardiomyocytes lost during infarction in rat [11].

On the contrary, vascularization techniques are beneficial in creating engineered heart tissue of relevance cell number. Vascularization is established through specially designed bioreactor that enables migration of endothelial cells from cell sheets integrating with the microchannel of bioreactor [49, 50]. Additional single cell sheet layers of 40 – 50 μm thick are added sequentially to form thick cell

sheet layer of 500 μm with vascularized network [50]. Based on mathematical calculation, the thick cell sheet construct amounted to 95×10^4 cells being transplanted into infarcted murine heart, a value close to the number of cardiomyocytes lost during infarction.

2.3.2.2 Cell seeding on prefabricated scaffolds

Conventional fabrication for engineered cardiac tissue uses solid scaffold as basis for cardiac cells attachment and mechanical support. These fabrication processes usually require two-step process, i) forming scaffold and ii) seeding cardiac cells. Several methods have been employed to construct scaffolds, namely, solvent casting [57-59], moulding [60], electrospinning [61-67] and selective laser sintering (SLS) [68]. One major disadvantages of methods such as solvent casting is the inability to produce uniform macropore structure in casted scaffolds. In subtractive fabrication method, porous structures are fabricated using laser ablation [69]. Comparatively, additive fabrication techniques such as SLS fabricate porous scaffold structure layer-by-layer [68]. Porous polycaprolactone (PCL) scaffold has been fabricated using SLS with stiffness of fabricated scaffold like native human myocardium (0.2 mPa). C2C12 myoblast seeded span across the interconnected pores of sintered PCL scaffold.

To control mechanical strength of engineered cardiac tissue while providing biologically attractive sites for cells attachment, Pok *et al.* casted multi-layered scaffold comprising of gelatin, chitosan and PCL into mould to form composite

disk with tensile strength in the range of (100 – 300kPa) [70]. Alternatively, electrospinning spins nanofibres to form scaffolds for subsequent cell seeding. This form of nanotechnology produces fibres resembling collagen fibres in human myocardium.

In electrospinning, the collector determines the orientation of fibre. Flat plate collector produces randomly aligned fibres, whereas rotating mandrel helps to align fibres in a singular direction [71-73]. Despite achieving appropriate fibre diameters through electrospinning, fibre alignment and mechanical properties of the native cardiac tissue could not be achieved [74]. A derivative method of electrospinning has been reported to produce scaffolds with controlled patterning. Melt electrospinning writing is a method that is based on electrohydrodynamic principle, where thin polymer strands are deposited onto a charged substrate. In the setup by Castilho, PCL and PCL blend fibres were produced in range of 3 – 12 μm [74]. However, this method fabricates fibres at a lower resolution as compared to electrospinning.

2.3.2.3 Casting of cell-hydrogel

Casting or moulding forms hydrogel and cells from a predefined mould. Several research groups have casted cell hydrogel encapsulate as potential for cell-based therapy and pharmacological testing [33, 75]. Collagen ring casted with cell suspension was grafted onto epicardium of rat heart where implantation of engineered ring construct forms compact heart muscles [75, 76]. Of recent study

conducted by Shadrin *et al.*, large cardiac patches of 36 x 36 mm were fabricated by casting fibrin-based hydrogel with human pluripotent stem cells [77]. Structures casted out of moulds are restricted by the design of master mould. The native structure of myocardium does not comprise of singular patterns. Instead, cardiomyocytes alignment varies across the transmural of myocardium [78].

2.3.2.4 Decellularized matrix

A technique that removes xenogenic cells from the matrix, leaving only network of extracellular matrix acting as mechanical support and biological factors, was repopulated through perfusion of desired cell type. Heart valves were the first cardiac components to be decellularized and repopulated with cells [79, 80]. Gradually, Harald and co-worker displayed repopulation of full heart organ from decellularized matrix [81]. However, perfusion of decellularized matrix is limited 70% of original volume. Homogeneous and high seeding cell density still remain elusive for repopulation of decellularized matrix [82]. Moreover, decellularized matrix repopulated with neonatal rat cardiomyocytes showed disorganized electrical propagation with disarray cardiomyocytes and decreased expression of Connexion 43 [83]. The different approaches in cardiac tissue engineering is compared in Table 2-1.

Table 2-1 Comparison of different fabrication techniques in engineered cardiac tissue

Technology	Applications	Strength	Weaknesses
Cells Only			
Cell Sheet Technology			
	<ul style="list-style-type: none"> Cell monolayer cultured on temperature responsive PNIPAm [55] Layered cell sheet onto perfusion reactor [49] Combining cardiac cell sheet with cells seeded in decellularized SIS [50] 	<ul style="list-style-type: none"> Intact cell-cell [Gap Junction] and cell-matrix junction Able to beat spontaneously and synchronously Vasculature network established for thick construct (300 – 800 μm) 	<ul style="list-style-type: none"> Difficult to handle thin layers of cell sheets
Technology	Applications	Strength	Weaknesses
Cells with Biomaterials			
Cell seeding on prefabricated scaffold			
Polymeric Scaffold	<ul style="list-style-type: none"> Neonatal rat heart cells seeded on laser microablation of accordion-like honeycomb microstructure of poly glycerol sebacate [69] 	<ul style="list-style-type: none"> Inherent structure guidance for cell orientation; Cell Alignment for effective contractile force Provide low in-plane resistance to contraction 	Non-homogeneous cell distribution through seeding

		<ul style="list-style-type: none"> • Tunable mechanical property with similar stiffness as native adult rat myocardium (54kPa) 	
Technology	Applications	Strength	Weaknesses
Cells with Biomaterials			
Cell seeding on prefabricated scaffold			
Electro-spinning	<ul style="list-style-type: none"> • Cardiac cells from neonatal Wistar rats on PMGI [62] 	<ul style="list-style-type: none"> • Nanofibre imitates ECM structure • Structural anisotropy; Cell Alignment for effective contractile force 	<ul style="list-style-type: none"> • Low macroporosity for cells to migrate and populate throughout electrospun structure • Non-homogeneous cell distribution through seeding
Casting of cell-hydrogel			
	<ul style="list-style-type: none"> • Embryonic cardiac myocytes, Collagen Type I, Matrigel casted into circular mould and subject to mechanical stretch [75] • Neonatal rat heart cells and fibroblasts casted into alginate scaffold containing Au Nanowires [84] 	<ul style="list-style-type: none"> • Striated shape • Electrical response • Undelayed electrical coupling • Prevented LV dilation • Improved mechanical properties • Calcium transient recordings at various sites 	<ul style="list-style-type: none"> • Difficult to handle thick tissue assembly

Technology	Applications	Strength	Weaknesses
Decellularized matrix	<ul style="list-style-type: none"> Removal of xenogenic cells from extracellular matrix and repopulate with native cells [81] 	<ul style="list-style-type: none"> Native extracellular matrix retained 	<ul style="list-style-type: none"> Sacrificial tissue is required to derive tissue Identification of ideal sacrificial tissue Inhomogeneous distribution of re-entry cells

2.4. Formation of cell aggregates for tissue engineering

Main motivation behind tissue engineering is to construct engineered tissue for reparation and restoration of organ functions. This requires delivery of engineered tissue with high cell density. An approach that has raised numerous interests is the formation of tissue spheroid [85, 86]. The benefits of tissue spheroid lie in the presence of cell-cell interaction and the resemblance to tissue growth in developmental biology [85]. Tissue spheroids can be formed either through i) Cell sedimentation at air-liquid interface ii) Cell sedimentation at concave and non-adherent surfaces iii) Continuous agitation of cell suspension. These correspond to conventional methods for tissue spheroids fabrication which are hanging drop method [87], non-adhesive micromoulding and rotary cell culture or spinning flask culture respectively (Figure 2-8 a – e) [88].

Figure 2-8 Spheroid formation techniques [A-D conventional spheroid formation techniques; E-G microfluidic-based spheroid formation techniques] a) hanging drop method, b) concave micromould, c) spinning culture flask, d) rotary cell culture, e) droplet formation of multiphase flow, f) array of microwells, and g) rotational flow in microchamber.

In hanging drop method, cells aggregate to form spheroid through gravitational force at the liquid-air interface. This method has several limitations. Aggregates formed are flat and disintegrate easily [89]. Also, the process of manual pipetting can lead to variations in droplet volume and cell concentration, causing the formation of polydispersed droplets. It is also a time consuming and low throughput method that is prone to human error. Media changes are needed to sustain the dissociation and growth of cells. Progresses are made to achieve long term hanging drop method. Lee *et al.* developed a hollow sphere structure through which culture medium can be continuously supplied to the droplet [90]. Modification to the hanging drop platform has been made to increase the

robustness of the droplets formed and the production of high throughput droplets [91].

Non-adhesive micromoulds are indentations that provide sites for spheroid formation after seeding and allow the continuous culture of cell aggregates. When arranged in arrays, the microwells can give high throughput and uniformly sized and shaped spheroids, which are controlled by the geometry of the microwell. The use of liquid overlay technique is critical in creating a non-adherent substrate for spheroid formation. Poly (ethylene glycol) is one of the substances that are used for grafting microwell surfaces. Generally, depending on the initial seeding density and microwell size, approximately 75% of the microwells are completely filled with cells after an overnight incubation, and half of the cells seeded end up aggregating and completely filling the space within the microwells [92]. Also, a pyramidal well facilitates the formation of a single spheroid in each well instead of several individual spheroids [93].

However, the mode of nutrient supply described in the conventional spheroid formation systems above is static. Alternatively, microfluidic system can be used to continuously supply nutrient during spheroid formation. Moreover, several performances such as cell isolation, cell concentrator and cell encapsulation can be integrated within a single microfluidic device. Additionally, harvesting units can be designed within microfluidic device for retrieval of cell spheroids. The

following describes different variation of microfluidic platforms for spheroids formation.

Droplet-based microfluidics form spheroids through cell encapsulation [94-96].

Microscale cell confinement facilitates cell-cell interaction and enables the formation of compact spheroids, and subsequent culturing for cell proliferation. This method produces high throughput of monodisperse multicellular droplets. Spheroid size is constrained despite cell proliferation by the encapsulation layers, whose size can be modulated by flow rate, channel geometry and fluid density. However, this method may need to be complemented with a cell concentration device as the current design only produces spheroids with low cell concentration. Also, cell entrapment within the droplet is subjected to Poisson distribution, which gives a range in terms of the number of cells within a droplet. Other issues include the need for the removal of the oil layer after encapsulation to prevent mass transfer impairment and ensure cell viability. Stress can lead to breakage of the membrane.

Micromoulding chambers in microfluidics Adaptation of non-adhesive micromould with microfluidic allows continuous supply of nutrient and removal of waste. In contrast to the conventional micromoulding method, micromoulds assisted with microfluidics were used to improve the efficiency of cell seeding and spheroid harvesting. Microchannels are used to direct flow of cell suspension into non-adhesive microchambers for cell spheroid culture [97]. Additionally,

various studies have investigated designs with continuous parallel flows for automatic cell seeding through hydrodynamic trapping [98-102]. In general, these methods rely on the interaction of forces such as gravity, buoyancy, drag and various fluid dynamic factors, depending on the flow conditions.

Numerical simulations were performed to determine the effect of input flow rate on particle seeding in the microwells in order to prevent particles from flushing out of the wells [98]. Aggregates were often observed within one day and high cell retention can be achieved at low input flow rate. The presence of a perfusion system allows long term culturing of tissue spheroids within the microwells. While these methods have the advantages of monodispersity and low cell loss, the reliance on gravity as the driving force for cell seeding suffers low spheroid formation rate. To counteract this shortcoming, flow-induced compression had been used [103]. However, this method can lead to the exertion of stress on cells, thus compromising cell vitality.

Attempts have also been made to address the inevitable need for spheroid harvesting. Flushing by aqueous solution had been performed and was shown to produce a 96.8% harvesting yield when performed at an appropriate flow rate [98]. However, there are others who argue that retrieval by this method produce low aggregate release and affect cell viability [104]. Stimuli-responsive materials such as PNIPAm had been investigated and they shed light on the possibility of creating a dynamic microwell environment for spheroid harvesting [104, 105].

High speed rotational flow for rapid formation of cell aggregates has been introduced and explored by many researchers [106-109]. This method consists of two parallel inlet channels with opposite flows to induce micro-rotation at high flow rates. While the obvious advantage of this method is the rapid formation of cell aggregates, which can be achieved in the order of a few minutes, it leads to large amount of cell loss. However, the cells may be reused, and care need to be taken to minimize the effect of shear stress from cell-cell and cell-wall interaction due to the rapid flow.

Physical barriers and forces for cell aggregation Physical barriers are designed within microfluidic device to trap cells in the direction of fluid flow. In designs that use semi-porous membranes, cell adhesiveness of membrane is altered by protein (adherent) and polymer (non-adherent) coating [110, 111]. The membrane is sandwiched between two layers of microfluidic channels. Pressure differences across the upper and lower microchannels induces cell attachment at porous opening of membrane. As membrane surface has been coated with non-adherent polymer, cells aggregate to form spheroids. U-shaped traps are patterned in microfluidic chips to capture and culture cells [103].

Externally induced physical force such as magnetic force can be used to aggregate cells when cultured in paramagnetic salt solution for cell assembly into spheroid at region of lower magnetic flux density [112]. In general, cells can be focused using external physical force before culturing into tissue spheroids. Table

2-2 summarises the cell-biomaterial manipulation using microfluidic design for tissue engineering applications.

Table 2-2 Cell-biomaterial manipulation using microfluidic design for tissue engineering applications

Tech-niques	Process	Advantages	Disadvantages	Ref
Droplet-Based	Microfluidic setup that contains multiphase flow (core and sheath) Cell suspension introduced at the core inlet flow	Rapid formation of cell-encapsulated droplet Easy to harvest through continuous flow	Static culture of aggregates Nutrient-waste exchange limited by diffusion process between inner capsule and outer media pool Low cell numbers captured within droplets Oil layer needs to be removed after encapsulation to prevent mass transfer impairment and ensure cell viability	[95, 113, 114]
Micro-moulding	Cell suspension introduced to an array of non-adhesive micromould Cells settled to the bottom of micromoulds through gravity	Continuous supply of nutrient to microwell Formation of spheroids within 24 hrs of culture	Requires coating of non-adhesive material Polydispersity in aggregate size which may be controlled with better well design Harvesting requires flushing of aggregates at high flow rate	[98-100, 104, 105, 115]

Tech-niques	Process	Advantages	Disadvantages	Ref
Rotational Flow	Rotational flow created within chamber creating different phases in cell suspension	Rapid formation process High throughput with arrays of chambers.	Requires coating of non-adhesive material	[106 - 109]
Physical Trap	Physical barriers in form of membranes or design features that capture cells in the direction of flow	Simple to set up feature	Capturing efficiency decreases with time as process is limited by the surface area of physical barrier available	[103]

2.5. Directing cellular alignment in 3D tissue cultures

In many human tissues, the complex anisotropic microstructure dictates biological and mechanical function. Cellular alignment is important when direction-dependent mechanical properties are essential in native tissues. For instance, electrical and mechanical properties of the heart [116], and multinucleation of muscle fibres in myotube formation for musculoskeletal tissues [117] requires high degree of cellular alignment for intended biological and mechanical functions. Aligned cells secrete highly organized, anisotropic extracellular matrix, triggering cascade of events in cellular pathway, which is essential in determining tissue function [118, 119]. There, it is essential to capture

the anisotropic properties within engineered cardiac tissue for better representation of the native myocardium.

Various studies have investigated the influence of mechanical and electrical stimulations in muscle differentiation and cell alignment. Topological (mechanical) and electrical stimuli were applied, independently or together, against muscle precursor cells to observe changes in cellular function such as cell orientation and alignment [120]. When both stimuli were applied together, the influence on cell differentiation was additive, with high Troponin I production, downregulation of Myogenin and upregulation of Desmin.

Other than changes in the cells' phenotype, electrical stimulation alone has impact on cell alignment. For instance, endothelial progenitor cells cultured in direct current re-orientates with their long axes perpendicular to the vector of applied electrical field [121]. Radisic *et al.* has established that myofibrils aligned in the direction of electric field lines [122]. The time point at which to apply electrical stimulation is critical in enhancing contractile behaviour.

Mechanical and electrical stimuli influence cellular function differently. For instance, topological cues largely influence Desmin upregulation whereas electrical stimulation down regulates Myogenin expression [120]. Down regulation of Myogenin is often presented in terminally differentiated muscle cells. However, the degree at which the influence of mechanical and electrical stimulation on cell alignment varies differently, with mechanical stimulation

inducing cell alignment. Cell aligned parallel with the surface pattern as compared to the direction of electrical stimulation, suggesting that topological cues have stronger influence on cell orientation as compared to electrical cues [123]. Hence, the principles of inducing cell alignment through topological cues, in general mechanical stimuli, are highlighted in the following section. Figure 2-9 highlights the mechanical cues that influence cell alignment.

Figure 2-9 Mechanical cues that influence cell alignment classified broadly into stretch-induced alignment versus geometric constraints.

2.5.1 Stretch-induced alignment

Static Strain induces cell alignment through structural changes in material when elongation/contraction of material is restricted. Both scaffold-based versus scaffold-free setup have been established to induce cell alignment. In scaffold-based techniques, there have been challenges in distinguishing whether the stimulus from materials' stiffness or strain factors dominates cellular behaviour such as cell alignment. To decouple the effect of materials' stiffness with stress/strain factors in influencing cell alignment, 10%w/v gelatin methacryloyl (GelMA) was formed into trapezoidal shape to create hydrogel substrate with strain gradient [119]. When the substrate is being pulled at one end, strain gradient is presented at different location of the trapezoid hydrogel. The strain pattern was simulated using COMSOL and were predicted to vary within the strain values where the stiffness of hydrogel is within the linear viscoelastic range. It was shown that cells seeded on GelMA surface with 20% strain demonstrated perpendicular alignment against the direction of stretch (stretch-avoidance). Whereas cells that are within the large strain area (17-19%) aligned perpendicularly, with parallel cell alignment in cells within lower strain range (13-15%). The cell alignment effect of the latter strain values was further confirmed through static stretching of rectangular GelMA substrates.

Similarly, several studies to understand the effect of static strain were designed to encapsulate cell-hydrogel between posts/frame. Cell alignment was

demonstrated to be in parallel with the direction of strain. For instance, when cell-collagen are encapsulated and restricted within posts demonstrated parallel alignment in the direction of strain on achieving maximal effective stiffness [124]. Human engineered muscle tissues seeded at 10^6 cells/mL of fibrin matrix was cultured between two silicon posts showed parallel alignment according to the strain direction [125]. The biological responses were enhanced with higher expression of adhesion molecules (10-fold increase in $\alpha 5$ integrin and 48% increase in vinculin) of 3D fibrin matrix as compared with 2D cultures.

Comparatively, cells have been encapsulated within moulds designed with posts to create constraining effect based on the shape of posts and the interdistance between posts. For instance, 10^6 cells/mL of isolated neonatal rat skeletal myoblasts mixed with Matrigel and fibrin hydrogels were seeded in Polydimethylsiloxane (PDMS) mould with post of varying length, width, and spacing [126]. The tissue bundle width was found to vary from 500 to 400 to 250 μm across post length of 0.6, 1.2, 1.8 mm respectively. It was found that with an increase in post spacing, there has been an increased in static strain which improves on local and global myofibres alignment. Despite reduced volume in engineered tissue of longer pores, the engineered muscle network due to synergistic effect between increase in cellular alignment and myonuclei number. Variation in the post length affects cell alignment, and tissue tension. After a threshold level in the degree of cell alignment is achieved (1.2 versus 1.8 mm post), an increased in the uniaxial tension enhances myofibres formation of cells

encapsulated against 1.8 mm post [126]. Enhanced myofibres formation was achieved through increased strain in bundle regions.

The above demonstrate cell-hydrogel remodelling process which induces cell alignment. In a similar setup, Bian and co-worker fixed casted cell-hydrogel construct against micropost of PDMS platform [127, 128]. Cell-hydrogel mixture that were formed with smaller struts (i.e. approximately 500 μ m from 1.2mm post) showed higher degree of cell alignment. It can be deduced that cell alignment is due to the constraining effect of smaller hydrogel struts when cell remodel the hydrogel by contraction. By restricting the contraction of cell-hydrogel struts using the micropost, there is a constant stress resisting along the struts direction. Similarly, gelatin methacryloyl struts measuring 50, 100 and 200 μ m were photopatterned to contain 10^6 C2C12 cells/mL demonstrated higher degree of cellular alignment for struts with lower dimensions [129].

Dynamic stretching aligns cell perpendicular to the direction of cyclic strain where the deformation rate is minimal [124]. Mechanical stimulation through stretching enhances myocytes function through upregulation of actin, Connexin 43, and N-cadherin factors in ventricle myocytes [130]. Stretch-avoidance is the main mechanism in cells that have undergone cyclic stretching. However, cell alignment due to stretch-avoidance can be overruled by contact guidance [124]. Cells aligned in the direction of constraint imposed using PDMS post at opposite

sides of collagen matrices (3-day static uniaxial constraint) did not realign when collagen matrix undergoes cyclic uniaxial stretch (3-day uniaxial stretching).

When encapsulated cells were analysed at three different planar sections, cells at surface did not demonstrate stretch-avoidance as previously expected from constructs undergoing cyclic straining [124]. Presence of fibrous collagen at core can explain the absence of stretch-avoidance for cells that are embedded within the core. Possible explanation could be due to the aligned collagen within core that limits cells at both bottom and top surfaces to align away from stretching. i.e. due to entanglement of cellular dendritic extensions with collagen fibrils. Hence, this demonstrates that contact guidance dominates over stress-fibre reorientation in view of stretch-avoidance response as described in uniaxial cyclic.

2.5.2 Geometrical constraints

Physical methods induce cell alignment through constraints created on substrate surface (microgrooves) or fibre scaffold for elongated cell growth. Anisotropic scaffold induces high order of cell alignment [60, 69]. For instance, Yeong *et al.* demonstrated laser ablation of PLLA-PCL scaffold to form channels with width and depth 100 μm [68]. C2C12 myoblast seeded on the scaffold showed degree of alignment in the longitudinal axis of channel. Laser ablation and moulding have been used for creating microgrooves topography for induced elongated cell morphology [60, 69, 70]. Astrocytes cells seeded within hollow cylindrical tubes of agarose, with collagen lined within the inner core, demonstrated higher degree

of alignment with smaller inner diameters of 180 and 350 μm as compared to 1 mm [131]. Curvature of substrate determines the degree of cell alignment with relative preference of cell spreading along direction of infinite curvature (along groove direction) when curvature of substrate is small (50 μm) [132]. Growing cells onto microgroove with PNIPAm coated surface produced aligned cell sheet [133]. The alignment induced by the geometrical constraints of microgrooves. aligned cell and produced cell sheet with anisotropic properties.

Alternatively, electrospinning produces nanoscale fibre structure where seeded cells demonstrated high level of alignment that ranges from 600 to 900 ηm [61, 62, 64-66]. The mechanism of cell alignment in electrospinning with nanoscale features differs from microscale alignment such as geometrical constraints as discussed above. Nanoscale stimulants regulate cell alignment via stimulating cell surface receptors that connects to cytoskeleton [134]. To increase bioactivity of electrospun fibres, hydrophilic materials such as silk fibroin have been blended into the electrospinning system to improve cell adhesion and proliferation ability on narrow fibres [135].

Inducing constant force on hydrogel prior to gelation can influence unidirectional growth of fibrillar network in collagen hydrogel. Collagen hydrogel showed directional fibrillar growth when balancing the process of fibrillogenesis versus force-induced matrix stretching [136]. Using a PDMS platform with anisotropic cavity, collagen pregel was loaded within the platform and subjected to constant

elongation upon released of the pre-stretched PDMS. It was found that a lag time of approximately 5 minutes is needed prior to stretching collagen pregel for aligning fibrillar network within the collagen matrix. The study continued to show cell aligned parallel to the stretch direction of collagen when encapsulated within matrix using the gelation process described above.

Biochemical methods, on the other hand, constraint cellular growth via selectively micropatterning growth factors onto substrate [134, 137]. Cells interact with the substrate via transmembrane integrin receptors that recognizes peptide sequences such as RGD, However, most of these discussed knowledges on cell alignment lies on cell seeding at substrate surfaces – rendering such process as 2D culturing of cardiomyocytes.

2.5.3 Current limitations

Cell alignment is guided by cell-material and cell-cell interactions. Several studies have mapped the factors that control cellular alignment as discussed above. Micro and nanotechnologies have been used to induce cell alignment for cardiac tissue engineering. Mainly two kinds of fabrication methods are observed, i) cell seedings on substrate and ii) cell encapsulation within hydrogel. However, alignment mechanism differs between cells seeded in 2D culture as compared to 3D culture. Distinctively, cell morphology in 2D differs with that of 3D culture [138]. Similarly, the response mechanism that induces cell alignment differs between the 2 culturing systems. For instance, cells seeded on 2D substrate

undergo strain/stretch-avoidance when uniaxially stretched and tend to align in direction perpendicular to principal strain [139]. However, cells that are cultured in 3D constructs (either encapsulated or seeded in scaffolds) respond differently with cells at gel surface that aligned in view of stretch-avoidance as compared to cells that are within the gel [124].

Fabrication methods to achieve aligned myocytes discussed such as dynamic/static stretching and biochemical patterning are beneficial in understanding cell-substrate interaction at planar level. The essence of cell alignment lies in conforming and guiding cells using defined spaces. Nanoscale features such as electrospinning guide cell alignment along spun fibres; Microscale geometries confine and direct cell growth towards anisotropic direction. However, these methods vaguely describe cell behaviour in native environment. To utilize such knowledge and integrate into a three-dimensional perspective requires engineered tissue with three dimensionality designs. Cyclic stretching is possible with planar setups whereby the directionality of alignment is bidirectional. However, it is difficult to alternate stress-fibre orientation in 3D constructs to effectively relate to the native myocardium. Hence, it is essential to modulate cell alignment in 3D microenvironment where contact guidance of ECM fibres in scaffolds impact stretch-induced alignment over time.

The ideal cell type used for engineering *in vivo* solutions to cardiovascular diseases has not been identified. Moreover, isolation of cells from endocardium,

epicardium and cardiac fibroblast have not been established [35]. While the ideal cell source will continue to pose as a limitation in engineered heart tissue, the focus of this research will remain on the method in fabricating an engineered myocardium.

With the high loss of cardiomyocytes during infarction, fabricating full thickness cardiac tissue has not been achieved mainly because of limited vascularization. The huge loss in number of cardiomyocytes requires a vascular network that facilitates nutrient transportation. Conventional methods in fabricating engineered heart tissue shows limited advancement in terms of vascularization.

2.6. Bioprinting for cardiac tissue engineering

2.6.1 Bioprinting technologies

In recent years, computer-assisted technology has been integrated into tissue engineering to develop biofabrication methods with controlled material deposition. Such technology is also known as bioprinting. Bioprinting, as described by Guillemot et. al., is “the use of computer-aided transfer process for patterning and assembling living and non-living materials with a prescribed 2D or 3D organization in order to produce bioengineered structures serving in regenerative medicine, pharmacokinetic and basic cell biology studies” [140]. This technology follows closely with its counterpart – additive manufacturing (AM). AM, also known as 3D printing, allows geometrical control over the placement of materials through computer-aided technology where it has been commonly used as scaffold building [68, 141, 142]. Scaffold built provide structural support and mechanical integrity for subsequent cell seeding.

However, these approaches have limited application in terms of soft tissues such as muscles and skins where modulus of soft tissue such as myocardium is in the range of 0.5MPa [37]. Engineered construct requires matching modulus with native tissue to allow integration. Moreover, cell seeding on scaffold has a major limitation in terms of heterogeneous cell distribution. An alternative is to incorporate cells during the fabrication process.

As described by Guillemot, bioprinting is the use of computer-aided transfer processes for patterning and assembling living and non-living materials with a prescribed 2D or 3D organization [140]. Using computer-aided technology, complexity of tissue can be mimicked through layer-by-layer deposition of viable living cells and materials with biochemical and physiochemical gradients. Figure 2-10 shows various methods used in bioprinting.

Figure 2-10 Classification of bioprinting technology Computer-aided bioprinting allows spatial control on the deposition of bioactive materials.

Material Jetting involves droplet displacement of material. Technology involving material jetting in bioprinting includes piezoelectric/thermal inkjetting, acoustic wave jetting, electrohydrodynamic jetting and laser induced forward

transfer [63, 143-146]. Cell density printed can be controlled by printing over a specific area while different cell types can be printed within a construct.

Laser-induced forward transfer (LIFT) uses pulsed laser source to deposit microdroplets of materials from a coated quartz ribbon to a receiving substrate. Thin films of metal, metal oxide or photo-decomposing volatile polymer are used for the interlayer. Polyimide layer is used instead to reduce the transfer of metallic materials onto biological constructs – blister-actuated LIFT [146]. Unlike orifice techniques, such as inkjet and extrusion, bioink used in LIFT is not restricted in its viscosity range. High cell loading density (10^8 cells/mL) can be printed using LIFT. Resolution of laser-based printed construct depends on bioink characteristic (viscosity and surface tension), laser pulse energy, focus spot diameter, and writing speed [147]. Gaebel and co-workers demonstrated printing of human mesenchymal stem cells (hMSCs) along with human umbilical vessel endothelial cells on Matrigel to yield cardiac patch with vessel-like structure [148]. Figure 2-11 shows schematic representation of the different jetting technology for depositing cells, biomaterials and biomolecules.

Figure 2-11 Schematic representation of the different jetting technology for depositing cells, biomaterials and biomolecules.

The printing does not require material deposition to be in close contact with receiving substrate. Hence, when different combinations of materials (cells, hydrogels and biological growth factors) are deposited from different loading inputs, there is lesser chance of cross contamination. In cases when the nozzle tips of precursor hydrogel and crosslinking agent, there is a chance of hydrogel gelation at tips hence clogging of nozzle.

Material Extrusion techniques extrude hydrogel from the nozzle, producing defined structures. Extrusion printing is generally powered by either a pneumatic [10, 149-157] or a mechanical pump [158, 159]. These pumps act by applying a

positive pressure on the hydrogel causing the gel to flow out of the nozzle. Additionally, valves can be placed at the nozzle to create droplets by regulating the flow of the hydrogel within the syringe [160]. A unique printing method is performed by Skardal and co-workers, where macrofilaments of hydrogel were first crosslinked into tubular constructs prior to printing [158]. Agarose hydrogel filaments are deposited first to act as support structures for subsequent cell-hydrogel filament. This conformal printing strategy can also be observed in hybrid printing of construct with thermoplastic filament adjacent to cell-laden hydrogel filament.

Optical Manipulation uses laser to optically manipulate material from one substrate to another [161]. This technique is also known as laser guided direct writing (LGDW). LGDW differs with LIFT where laser causes an axial pushing force to control and displace individual cells (Figure). Patterned hepatocytes and endothelial cells was constructed with single cell resolution [161]. Figure 2-12 shows the differences between laser guided direct writing and laser induced forward transfer.

Figure 2-12 Differences between (left) laser guided direct writing (right) laser induced forward transfer.

Vat Polymerization Printing (VPP), a container filled with cell-hydrogel suspension is subjected to UV light curing to form 3D structures. Hydrogels used in this printing system are light sensitive photopolymers such as poly(ethylene glycol) diacrylate (PEGDA) and gelatin-methacryloyl (GelMA). Three dimensional constructs are formed point by point through laser curing in stereolithography (SLA). Alternatively, UV light are area-projected in digital light processing (DLP) into the vat of photopolymer using digital micro-mirror device. Soman and co-workers demonstrated the robustness of DLP system through encapsulating cells within various geometries [162]. Deformation of geometries shows that cells were remodelling the surrounding matrices. Figure 2-13 shows stereolithography and dynamic optical projection used in bioprinting.

Figure 2-13 Schematic representation of stereolithography (left) and dynamic optical projection (right).

Fedorovich and co-workers demonstrated that photo-exposed mesenchymal stem cells resulted in damages of cells at DNA level [163]. However, this is a general limitation in using photopolymer such as PEGDA and GelMA. Yet not all cell types, such as fibroblast cell lines, display such sensitivity towards photo-exposure [163]. Table 2-3 compares the different bioprinting techniques.

Table 2-3 Comparison of different bioprinting techniques

Techniques	Resolution	Advantages	Disadvantages
Material Jetting			
Piezoelectric/ Thermal	100 µm	<ul style="list-style-type: none"> • Picolitre droplets gives high resolution • Contact-less printing minimizing cross contamination risk 	<ul style="list-style-type: none"> • Limited to low viscous materials • Limited control over printed microstructure
Electro Hydrodynamic Jetting	10-20 µm		
Acoustic Droplet Ejection	37-150 µm		
LIFT	10 – 100 µm	<ul style="list-style-type: none"> • Single cell resolution • High cell loading density (10^8 cells/mL) • Any viscosity of loading material 	<ul style="list-style-type: none"> • Long preparation process, requires pre-fabrication of cell-coated ribbon • Complex instrumentation for precise control needing materials with optical property.

Material Extrusion			
Pneumatic and Mechanical	15 – 400 μm	<ul style="list-style-type: none"> • Wide choice of materials • Able to control material extrusion by modifying needle tip • Allows control over microstructure of printed biomaterial 	<ul style="list-style-type: none"> • Resolution limited by cell viability • Clogging of needle tip
Techniques	Resolution	Advantages	Disadvantages
Optical Manipulation			
Laser Guided Direct Writing	100 μm - 10 μm	<ul style="list-style-type: none"> • Single cell resolution 	<ul style="list-style-type: none"> • Slow process • Energy directed towards cells
Vat Polymerization Printing			
Stereolithography (SLA)	~1 mm	<ul style="list-style-type: none"> • High resolution 	<ul style="list-style-type: none"> • Limited cell encapsulated • Cell damaged through photo-exposure
Digital Light Processing (DLP)	20 – 200 μm		

2.6.2 Biomaterials for bioprinting

Biomaterials, specifically hydrogel, have been widely used in bioprinting. Hydrogel, a three-dimensional polymeric substance, can retain large quantities of water, has been widely used in the field of tissue engineering and regenerative medicine. Hydrogel is either printed as a scaffold where cells are seeded thereafter or used as a carrier for cell deposition in bioprinting. Types of hydrogel that have been commonly used in bioprinting includes naturally occurring collagen [63, 164, 165], gelatin [10, 166], fibrin [165], alginate [154, 167, 168], chitosan [169], and hyaluronic acid [158, 170]. Synthetic hydrogel such as polyethylene glycol [158, 171, 172], Pluronic F127 [173], and chemically modified gelatin [150, 174] have been used as carrier for depositing cells via bioprinting. Increasing research has been developing bioink derived from decellularized extracellular matrix (dECM) [175, 176]. The main advantage of using dECM as the bioink for printing is the presence of growth factors that may stimulate desired cellular responses. However, extracting large scale of dECM-based bioink is not feasible. Hence, one of the promising hydrogel that has been commonly used in bioprinting is the chemically modified form of gelatin – gelatin methacryloyl (GelMA).

GelMA has been widely used as the material for bioprinting [150, 177]. As an engineered ECM-based matrix, GelMA possesses tunable properties while maintaining cell-binding moieties [178]. Unmodified gelatin is physically

unstable at culturing condition. However, the methacryloyl group in GelMA makes gelatin photopolymerizable, forming a stable hydrogel which can be incubated at 37°C. Although other crosslinking methods (glutaraldehyde and transglutaminase) have been explored to form physically stable gelatin hydrogel at culturing condition, the reagents used for crosslinking are toxic to cells [179].

Colloidal science, crosslinking mechanisms and ionic interactions are some of the basic principles behind hydrogel printability in bioprinting [180]. Bioprinting controls material deposition at microscale level. Modular setup in bioprinting enables multi-material deposition through increasing number of printheads. Microscale deposition enhances hydrogel formation due to reduction in diffusion distance between hydrogel precursor and its crosslinker [181]. Hydrogel with fast crosslinking mechanism such as fibrin and calcium alginate are chosen and designed to deposit precursors and crosslinker sequentially [165, 181, 182].

2.6.3 Bioprinting strategies for 3D bioprinting of cell-hydrogel

Various printing and crosslinking strategies have been used to achieve print fidelity and resolution. In many cases, one or more of the strategies were used in the bioprinting process to achieve structurally stable bioprinted construct (Figure 2-14). To print a 3D bioprinted construct with structural integrity is a balance between biomaterial's rheology and designing the printing toolpath for biomaterials and cells deposition.

Figure 2-14 A bioprinting process that uses combination of different printing strategies to improve print fidelity and resolution.

Direct Bioprinting

Materials are printed directly to form the pre-determined shape and configurations. Materials or bio-inks formulations are optimized to achieve desired viscosity, yield stress and mechanical integrity for fast shear recovery during and after printing [183-185]. Some methods of optimization for direct bioprinting includes forming semi-crosslinked mixture and adding thickening agent [29, 168, 177, 183, 186-191]. Adding enzyme crosslinker [189], adjusting temperature of thermosensitive hydrogel [190] and pre-exposure of photopolymer to UV light [29] are some methods to improve viscosity of bio-ink through partially crosslinking the hydrogel. For instance, when Skardal *et al.* exposed the photopolymer to UV light prior to printing, viscoelasticity of the hydrogel was improved to equate the storage modulus G' to the loss modulus G'' for better extrusion [29]. Also, using thickening agents such as hydroxyapatite particles [183, 187], nanocellulose [168], xanthum gam [186], and gellan gum [183] helps to improve the printability of bio-ink to achieve a suitable viscosity for extrusion bioprinting.

In-process Crosslinking

Hydrogel with rapid gelation mechanism, such as alginate and fibrin, are commonly used through the in-process crosslinking method. In-process crosslinking is achieved by either modifying the extrusion head for coaxial extrusion of hydrogel precursor and crosslinker or depositing precursor into crosslinker bath [192, 193]. Alternatively, precursor hydrogel and crosslinker are sequentially deposited via designing the printer's toolpath [165, 181, 182, 194].

Post-process Crosslinking

In many cases, such printing approach uses mixture of hydrogels with multiple crosslinking mechanism. The mixture comprises of a primary material that improves printability and shape fidelity during printing while the secondary material later undergoes crosslinking to provide structural fidelity after printing [10, 149, 177]. For instance, gelatin-methacryloyl (GelMA)/hyaluronic acid (HA) mixture showed enhanced printability as opposed to using GelMA alone [177]. HA improves the viscosity of mixture while crosslinking of GelMA retains structure's integrity after printing.

Support-assisted bioprinting

Build/support configuration is commonly used to bioprint construct with high shape fidelity. Build materials refer to the intended engineered tissue component (i.e. cells and/or hydrogel), while support materials provide mechanical strength

to hold the structure. Whether the support material is deposited relatively with build material [29, 188, 195-198] or exists as a bath [199, 200], the support materials is removed through post processing in most cases. Hydrogel crosslinker can be incorporate into the support bath to enhance print fidelity while sacrificial bath is removed [199]. Hydrogels with reversible crosslinking mechanism such as gelatin, Pluronic F127, and agarose are commonly used as the support material.

Hybrid Bioprinting

The use of computer-assisted technology in bioprinting allows integration with several other fabrication processes. For instance, bioprinting techniques (such as extrusion and inkjet) has been integrated with melt-plotting and electrospinning apparatus to build multiscale parts [18, 63, 153, 155, 197, 201, 202]. Thermoplastic PCL and poly (lactic-co-glycolic acid) (PLGA) are commonly used as support material for hydrogel in hybrid printing. These thermoplastics are processed at high temperature which is not compatible to include cells in the plotting process. Instead, cells are deposited onto the thermoplastic using hydrogel as the carrier. Table 2-4 summarizes the different bioprinting strategies in achieving 3D construct.

Table 2-4 Summary of different 3D bioprinting strategies

Strategies	Method	Bioprinting Techniques	Application	Ref
Direct bioprinting	Optimizing viscosity via semi-crosslinked precursor, use of thickening agent	Extrusion	Cartilage	[168, 177]
			Skin	[29, 186-191]
In-Process Crosslinking	Pre-mixture of precursor with crosslinker (Co-extruder)	Extrusion	Lumen construct for nutrient delivery	[154, 193, 203] [149]
	Deposition of precursor into crosslinker	Extrusion	Femur, Arteries, Heart, Brain	[199]
		Inkjet		[192]
	Deposition of precursor and crosslinker sequentially	Microvalve		[181]
Post-Process Crosslinking	Expose printed construct to crosslinker after printing	Extrusion	Aortic Valve	[10]
			Cartilage	[177]
			Lumen construct for nutrient delivery	[10]
Support-assisted Printing	Printing of bio-ink with support mould or within a support bath	Extrusion	Vasculature	[195, 196, 200]
			Femur, Arteries, Heart, Brain	[199]
		Inkjet		[29, 188]
			Vasculature	[63]
Hybrid Printing	Cross-technology deposition of bio-ink and scaffolding material	Extrusion		[204]
			Bone, Cartilage, Muscle	[197]
		Inkjet	Cartilage	[153, 176]. [205]

2.6.4 Bioprinted cardiac construct

Conventional fabrication processes discussed in the earlier section mostly describe planar construction of engineered heart tissue. However, several research groups are using bioprinting to produce engineered cardiac tissue/structure to construct three dimensional structures with heterogeneous cell population [10, 206]. The potential in using 3D bioprinting technology for fabricating engineered cardiac tissue is in the control of the geometrical shape or anatomically relevant structures. As demonstrated by Duan, aortic valve conduits were built using different material by an extrusion-based bioprinter [10]. Another significant bioprinted construct was the vasculature network demonstrated by Kolesky and co-worker [195]. This potentially provides solution for vascularization in engineered heart tissue.

Gelatin methacryloyl mixed with gold nanoparticles were printed using a coaxial nozzle (ID: 200 μm OD: 600 μm) [207]. Cardiac fibroblasts (CFs) and cardiac muscle cells (CMs) were mixed into the silver and GelMA hydrogel mixture prior printing. Heterogenous cells comprising of harvested neonatal rat cardiac fibroblasts and cardiac cells exhibited low Young's modulus of 4.2 kPa with resemblance to neonatal rat heart tissue of 4 – 11 kPa, and adult rat tissue of 11.9 – 46.2 kPa. Gold nanoparticles increased electrical conductivity of the hydrogel material, which enhanced cell-cell connection, while slowing the proliferation rate of CFs cells to prevent from overpopulating the construct. Approximately

250 μm cell-hydrogel struts were created into a 0° - 90° lattice construct. Table 2-5 summarises the techniques used for some of the bioprinted cardiovascular and cardiac-related tissue.

Table 2-5 Bioprinted cardiovascular and cardiac-related tissue

Technique	Cells	Biomaterials	Application	Ref
Cell laden alginate/gelatin gel extruded into disc mould and cross linked in CaCl ₂	Porcine aortic valve interstitial cells Human aortic root smooth muscle cells	Gelatin Type A and alginate dissolved in sodium chloride solution	Heterogeneous aortic valve conduits	[10]
Cell/Alginate construct extruded and later polymerized CaCl ₂	Human fetal cardiomyocyte progenitor cells	Sodium alginate dissolved in culture medium	Tissue model	[206]
Sequentially extruding multiple inks such as Pluronic F127 and cell-based GelMA	Human neonatal dermal fibroblasts Human umbilical vein endothelial cells (HUVECs) 10T1/2 fibroblast cells	Silicone elastomer with base for border printing Pluronic F127 Gelatin methacryloyl	Vascular Branches	[195]
Cylindrical cell aggregates extruded into non-adhesive Teflon or agarose moulds	N-Cadherin transfected Chinese Hamster Ovary cells Human umbilical vein smooth muscle cells Human skin fibroblasts	Agarose	Vascular Tubular Grafts	[208]
Cells transferred onto Polyester Urethane Urea cardiac patch via LIFT	Human mesenchymal stem cells (hMSCs) Human umbilical vein endothelial cells	Polyester Urethane Urea Cardiac Patch	Cardiac Patch with different cell populations	[148]

Technique	Cells	Biomaterials	Application	Ref
Perpendicular hydrogel struts and transplanted	cell- printed cells	Human fetal cardiac-derived progenitor HyStem matrix	Cardiac grid transplanted into mouse model	[209]

2.7. Research methodology

In the previous section, numerous fabrication techniques have been described to achieve aligned myocytes. However, most of the constructs fabricated demonstrated planar alignment at a single plane. The alignment at planar level was understood as a form of cell-substrate interaction where geometrical cues from substrate conform and guide cells to grow in anisotropic direction using defined spaces. Both geometrical cues and mechanical stimulation, such as bidirectional cyclic stretching, are unable to attenuate 3D stress-fibre orientation that mimics the native myocardium. 3D bioprinting serves as the potential tool for fabricating engineered constructs with directional cell alignment. In this research, extrusion-based bioprinting system will be used to deposit hydrogel struts that induce cell alignment along print path for engineering cardiac tissue.

2.7.1 Influence of printing parameters and material property on print resolution of extrusion-based bioprinting

Controlling the print definition is essential in bioprinting for cardiac tissue engineering. The relation between printing parameters and material properties with print resolution is essential in obtaining printed hydrogel dimensions. Generally, the dimension of bioprinted construct is limited due to the diffusion limits of oxygen and nutrients into cell-encapsulated hydrogel.

2.7.2 Material formulation for bioprinting of cell-hydrogel through extrusion

A material selection framework is necessary to establish steps for material evaluation when determining the suitable bioink composition for bioprinting. The materials' properties must satisfy both cell-based and process-specific requirements. This material selection framework serves as a platform framework and is applicable for different bioink and printing process. Extrusion-based bioprinting was chosen and hence, the effect of shearing on cell survivability is essential for evaluation.

2.7.3 Support-assisted bioprinting for directing cell alignment

Various printing strategies have been described to improve the shape fidelity of 3D bioprinted constructs. Of which, support-assisted bioprinting enables deposition of material with wide range of viscosity without comprising the fidelity of print. Hence, it is essential to understand the process of support-assisted bioprinting and control the geometry of deposited cell-hydrogel for inducing cell alignment. Cell alignment induced via extrusion-based bioprinting is characterised. The alignment of cells along the printed struts is postulated to resembles cell alignment of the myotubes.

2.7.4 Enabling technologies for 3D cardiac organ printing

Pre-printing processes such as print file preparation and processing cells for printing are investigated. Data processing in terms of multi-material bioprinting and the use of coordinate-based control over material and cell deposition has been studied. By mimicking the fibre orientation of native myocardium, print paths are strategically designed to control material deposition following the contours of the native tissue. 3D printed microfluidic devices are characterized and evaluated on the feasibility of printing a complex 3D microfluidic device for fabricating cell aggregates. The research methodology is shown in Figure 2-15 Research methodology.

Figure 2-15 Research methodology that investigate on the bioprinting process in i) controlling print resolution and definition ii) material formulation.

Chapter 3. Effect of printing parameters and material properties on print resolution of extrusion-based bioprinting

3.1. Introduction

The extrusion-based bioprinting system has been adopted from manufacturing industry namely, electronics packaging, pharmaceutical dispersions, and food processing [210, 211]. Materials in paste and powder form are mainly used in pharmaceutical and food processing technology [210, 211]. Distinctively, extrusion-based printing can be divided into three categories i) positive displacement ii) time-pressure dispensing iii) rotary-screw extrusion (Table 3-1). Mechanisms for extruding material differ between these systems. Positive displacement uses motor to drive piston downwardly, hence extruding material based on rate of motor's displacement; Time-pressure dispensing system supplies air pressure into the syringe cartridge to extrude material, where the difference between supplied air pressure and ambient pressure drives the flow of material; Rotary-screw extrusion with a motor-drive screw, displaces material through the nozzle. Speed of motor displacement determines the amount of material dispensed in both positive displacement and rotary screw systems – mechanically different from the time-pressure dispensing system.

In extrusion-based bioprinting, both pneumatic [10, 149-157] and mechanical systems [158, 159] have been commonly used for dispensing cell-hydrogel. However, only time-pressure (pneumatic) and positive displacement [154, 155, 212] extrusion technique has been employed in bioprinting of cell-laden hydrogel. The use of rotary screw extrusion in bioprinting has been limited only when high viscous material such as PCL melt is used [159]. These techniques allow processing of materials with a wide viscosity range ($30\text{mPa/s} - 6 \times 10^7\text{mPa/s}$) [213]. An advantage of using extrusion-based bioprinting system is the customizability of extrusion nozzle/needle. Several studies have designed nozzles for co-extrusion of materials to increase printability of soft material [154], produce hollow hydrogel fibre resembling blood vessel [149] and deposit cell aggregates [214]. As such, even high cell density aggregates can be printed using this technique [215].

Research has been carried out to understand the fluid dispensing process in electronic packaging in terms of i) characterization of flow behaviour ii) representation of flow rate of fluid dispensed iii) profile of fluid dispensed on electronic board [216]. Specifically, modelling of positive displacement, pneumatic, and rotary screw dispensing system has been investigated by Chen and co-worker for electronic packing [217-219]. The flow behaviour of dispensing fluid has been commonly described using generalized power law, where the power law index (n) and consistency index (K) are obtained through rheological data of fluid. Extrusion-based bioprinting shares close resemblance

to electronic packaging where the setup involves dispensing Non-Newtonian fluid of similar viscosity onto a substrate surface.

Several studies optimize time-pressure bioprinting which provide ground work for relating effect of printing and material parameters with cell viability and formation quality [187, 220, 221]. Additionally, the ability to control printed hydrogel width is essential in bioprinting. When cells are encapsulated within hydrogel, there is a 100 – 200 μm diffusion limit for exchange of oxygen and nutrients [222]. Hence, it is essential that dimension of printed cell-hydrogel should not be more than 400 μm in width. This renders the need to understand extrusion process to identify printing parameters and material properties in influencing print resolution.

This section reports the various factors that affect print resolution of a time-pressure dispensing system. Factors such as pressure, moving speed, viscosity of material and material-substrate interaction are investigated. Alginate is used as an analogous material to identify rheological behaviour of hydrogel and its correlation to the profile of material dispensed and evaluate on the ability to achieve three-dimensional structure based on the mechanical properties of hydrogel. This section serves as a platform in identifying factors or printing strategies that produce controlled cross-sectional geometry of extruded material for bioprinting with shape fidelity.

Table 3-1 Comparison of different extrusion systems

	Positive Displacement (Mechanical)	Time-Pressure (Pneumatic)	Rotary Screw
Factors affecting Flow Rate	<ul style="list-style-type: none"> • Position of piston • Speed of piston movement • Fluid compressibility • Fluid behaviour 	<ul style="list-style-type: none"> • Needle geometry • Pressure applied • Fluid flow behaviour 	<ul style="list-style-type: none"> • Motor speed and degree of rotation • Applied pressure • Flow behaviour of fluid dispensed • Screw geometry
Advantages	<ul style="list-style-type: none"> • Volume of material displaced not affected by time 	<ul style="list-style-type: none"> • Easy to set up 	<ul style="list-style-type: none"> • Fluid of high viscosity can be dispensed at high flow rate
Disadvantages	<ul style="list-style-type: none"> • Flow rate of material varies with position of piston 	<ul style="list-style-type: none"> • Volume of material dispensed changes with time 	<ul style="list-style-type: none"> • Difficult to control amount of material dispensed

3.2. Materials and methods

3.2.1 Hydrogel preparation

To investigate the effect of rheological properties of hydrogel on printing resolution, different composition of sodium alginate: calcium chloride was used. 6 and 12 g of sodium alginate powder (Sigma Aldrich) was dissolved in 10 mL of deionized water. 1, 2, 3 g of calcium chloride (Sigma Aldrich) was dissolved in 10 mL of deionized water. Solutions with different concentration were mixed as according to the volume ratio sodium alginate: calcium chloride, 7mL: 3mL as shown below. Mixture was left to stand under room temperature overnight to stabilize the gel before further analysis.

3.2.2 Rheological testing

Rheological study was performed on three different concentrations of alginate hydrogel using parallel plate rheometer (DHR, TA Instrument). Dynamic viscoelastic behaviour of alginate hydrogel was measured using a parallel plate rheometer (DHR, TA Instruments). A 40 mm diameter parallel plate measuring geometry with gap 500 μ m was used to perform an amplitude sweep under oscillatory mode. Constant frequency of 1Hz was set with logarithmic increase in stress ranging of 0.001 – 1000 Pa. Storage modulus (G') and loss modulus (G'') was recorded for three samples for each concentration. Flow sweep shear rate

between 1 to 1000 rad/s was also performed to investigate the viscosity profile of alginate hydrogel with varying composition.

3.2.3 Printing parameters

Time-pressure dispensing system (Biofactory®, RegenHU, Germany) was used for this study. To investigate a range of printing parameters that are suitable for both low and high viscosity alginate hydrogel, stage speed was held constant from the range of 800 to 2000 mm min⁻¹. Pressure was adjusted according to the composition of hydrogel studied to produce filament when printing. Needle with 250 µm inner diameter and average needle length of 10 mm was used.

Additionally, to investigate the spreading of hydrogel in different medium, a study has been done to understand hydrogel-substrate interaction. Hydrogels were printed onto different substrate surfaces with varying hydrophobicity. Glass slide and poly (methyl methacrylate) (PMMA) were chosen to understand the effect of hydrophobicity with respect to printed construct width. Water contact angle was observed through a sessile drop of deionized water using Theta optical tensiometer. Water contact angle on glass slide and PMMA are measured as 27° and 74° respectively. The degree of spreading effect of material was assessed by

calculating the difference between construct width printed on glass slide and PMMA using the following equation

$$\% W_{\Delta} = \frac{(W_{PMMA} - W_{Glass})}{W_{Glass}} \times 100\% \quad \text{----- (3.1)}$$

To investigate on print resolution in time-pressure dispensing system, four lines were printed. The images were captured using an inverted microscope (Olympus DP70) and analysed using ImageJ. A total of ten readings were recorded for each print parameter setup.

3.2.4 Mathematical model of fluid behaviour in extrusion-based bioprinting

In a time-pressure dispensing system, fluid's flow behaviour can be described through balancing of forces of a finite volume. Within the needle, the forces acting on the finite volume (dotted) is illustrated as below (Figure 3-1)

Figure 3-1 Resultant forces acting on a finite volume within a cylindrical geometry.

$F_Z = 0 = F_1 + F_2 + F_3$, where F_1 is the force driven by pressure difference, dZ defines the length of element. Shear stress τ_r is described as

$$\tau_r = \frac{r}{2} \cdot \frac{\partial P}{\partial Z} \quad \text{----- (3.2)}$$

$$\dot{\gamma} = \frac{dv}{dr} \quad \text{----- (3.3)}$$

For this study, Power Law model was used to describe Non-Newtonian flow behaviour for the hydrogel.

$$\tau = k \dot{\gamma}^n \quad \text{----- (3.4)}$$

$$\eta = \eta_o \left(\frac{\dot{\gamma}}{\dot{\gamma}_o} \right)^{n-1} \quad \text{----- (3.5)}$$

where n is the power law index, η_o is the initial viscosity, $\dot{\gamma}$ and $\dot{\gamma}_o$ are the shear rate and reference shear rate respectively. Assuming reference shear rate $\dot{\gamma}_o = 1 \text{ s}^{-1}$,

$$\eta = \eta_o(\dot{\gamma})^{n-1} \quad \text{----- (3.6)}$$

To identify volumetric flow rate Q of Non-Newtonian fluid in a simple cylindrical geometry, it can be determined as follows

$$V_o = K \left[\frac{n}{n+1} \left(R^{\frac{n+1}{n}} \right) \right] \quad \text{where } K \text{ is a function of } \frac{1}{\eta_o} \quad \text{----- (3.7)}$$

$$Q = \pi R^{\frac{3n+1}{n}} \left(\frac{n+1}{3n+1} \right) \left(\Delta P \cdot \frac{1}{2\eta_o} \right)^{\frac{1}{n}} \quad \text{----- (3.8)}$$

Mass flow rate of hydrogel is calculated by determining the density of hydrogel, ρ as follows $Q_m = Q \times \rho$ ----- (3.9)

Mass flow rate for each alginate hydrogel was recorded by weighing the amount hydrogel dispensed within a set time frame at each pressure interval. Volumetric flow rate of alginate hydrogel was calculated based on equation 8, assuming density of hydrogel is close to density of water ($\rho = 999.97 \text{ kg/m}^3$).

The second part of the modelling is to identify the width of the printed hydrogel. The width of hydrogel strand printed can be correlated to its volumetric flow rate Q and speed of the moving stage V through conservation of volume.

$$\frac{Q}{2V_m} = \int_0^a h(x) dx \quad \text{----- (3.10)}$$

3.3. Results and discussions

3.3.1 Effect of polymer concentration on viscosity and viscoelasticity of material

The viscosity profile of varying composition of alginate hydrogel is shown in Figure 3-2. The degree of shear thinning varies between alginate of different composition. As the alginate concentration increases from 6% w/v to 12% w/v there is a substantial change in the gradient of the viscosity vs shear rate graph. Similarly, alginate hydrogel of varied crosslinking density (12:1, 12:2, 12:3) showed an increase in the slope of viscosity profile when the crosslinking density increases. Cross-referencing between Figure 3-2A and B, higher crosslinking density yield construct with higher storage modulus values ($12:3 > 12:2 > 12:1$) which give rises to a change in the viscosity profile.

Through the rheological data, several observations can be made. The degree of crosslink affects the rate at which the hydrogel undergoes shear thinning process. As the concentration of alginate increases from 6%(w/v) to 12%(w/v), its zero viscosity and power law index increases. It is explained through an increased in polymer chain at higher mass concentration (12% w/v). Viscosity value increases as the concentration increase [223].

Figure 3-2 Rheological data of alginate hydrogel (A) Viscosity profile of alginate hydrogel with varying concentration (B) Amplitude sweep of alginate with different concentrations.

In the current study, calcium chloride was added to sodium alginate prior to printing. The hydrogel was stabilized overnight at room temperature prior to

loading the semi-crosslinked hydrogel for experiments. This is one of the printing strategies identified to enhance hydrogel's printability (Table 3-2) [184]. The first was to increase the polymer concentration aiming to increase its viscosity. In the second strategy, hydrogel is cross-linked prior to loading of printer's cartridges.

With increasing concentration of alginate used, the formation of extrudate varies. Mainly alginate hydrogel at lower concentration (6:1) produce droplets when dispensed whereas alginate hydrogel at higher concentration (12:1, 12:2) produces continuous flow of hydrogel upon dispensing. Relating to Schuurman's study on GelMA and gelatin-methacryloyl/hyaluronic acid (GelMA/HA) mixture, GelMA forms droplet upon dispensing whereas GelMA/HA dispenses continuous strands of hydrogel [177]. Sol-gel transition of hydrogel should be accounted for during printing process. Hydrogel in its uncross-linked form (precursor) has low viscosity and forms droplets instead of strand during deposition [177]. Relating to the rheological profile in Figure 3-2, the increased in viscosity and viscoelasticity of hydrogel improves printability of hydrogel.

Table 3-2 Comparison between two extrusion-based printing strategies for improved printability

	Strategy 1	Strategy 2
Before Printing (Zone 1)	Precursor hydrogel with increased polymer concentration (solution with higher viscosity)	Crosslinked hydrogel (gel with solid-like behaviour)
During Printing (Zone 2)	**shear thinning**	**shear thinning**
After Printing (Zone 3)	Crosslink factor added to form hydrogel	Hydrogel recovery from shear thinning
	<p>The diagram illustrates the three zones of extrusion-based printing for two strategies. Zone 1 (Before Printing) shows a precursor hydrogel solution (Strategy 1) and a crosslinked hydrogel (Strategy 2). Zone 2 (During Printing) shows shear thinning for both. Zone 3 (After Printing) shows crosslink factor addition (Strategy 1) and hydrogel recovery (Strategy 2).</p>	

3.3.2 Effect of printing parameters on print resolution

To understand the relation between processing parameters such as applied pressure and moving speed with print resolution, printing studies were conducted using 12% w/v alginate. The width of hydrogel struts printed was plotted against the moving stage speed. As shown in Figure 3-3, the moving stage speed shows a non-linear relation with respect to width of hydrogel struts deposited. Using the above graph, estimation can be made such that using alginate 12:1 with moving speed = 5000 mm min^{-1} , and pressure = 3 bar, W_c of $800 \text{ }\mu\text{m}$ can be constructed.

Figure 3-3 Graph showing the non-linear relation between moving stage speed with dimension of construct width of different alginate composition A) 12:1 B) 12:2 C) 12:3.

The highest resolution of $570\text{ }\mu\text{m}$ is achieved with 12:2 alginate hydrogel at a speed of 5000 mm min^{-1} using 3.5 bar. Understanding the printing parameters allow control over the geometry of construct. As such, a grid shape with controlled pore size of approximately $750\text{ }\mu\text{m}$ was constructed. Draw path of the construct is as shown in Figure 3-4. The averaged measurement of W_P from Figure 3-4 was $683.18\text{ }\mu\text{m}$, with 91% accuracy.

Figure 3-4 Controlling pore size of grid printing (white scale bar: $500\text{ }\mu\text{m}$).

PMMA surface is more hydrophobic than glass slide. By varying the substrate used for studying material-surface interaction, it was found that with a change from a more hydrophilic surface (glass slide) to a more hydrophobic surface (PMMA), there is a general decrease in the construct width. Additionally, there is a reduction in the percentage change of hydrogel width when there is an increase in the concentration of calcium chloride from 12:1 to 12:3 (Figure 3-5).

Figure 3-5 Percentage change of hydrogel width on different substrate.

The effect of material-substrate interaction on printing resolution was also investigated in inkjet cell printing system [224]. It was found out that the stiffness of substrate has an impact on the printing resolution through the absorption of impact force during inkjet printing. The understanding of deposited material on substrate is relevant when building construct with height; where the width of material deposited interacts with the previous deposited layer.

3.3.3 Mathematical model relating process parameters with print resolution

To develop the mathematical model that relates printing and material parameters with print resolution, the cross-sectional profile of printed hydrogel should be accounted for. Two cases may arise where the area under $h(x)$ can be described as either a circle or a half ellipse as shown in Figure 3-6 below.

Figure 3-6 Cross-sectional area of hydrogel struts printed A-C) ideal extrusion based bioprinting toolpath with circular cross-sectional area D-F) extrusion based bioprinting toolpath with elliptical cross-sectional area G) mathematical equation describing the different cross-sectional area.

To understand the width of the hydrogel obtained, the height between needle and platform was fixed at 0.1mm for analysis. By keeping the height constant, various platform's moving speed was investigated. Width of hydrogel printed is measured using ImageJ software. A fixed time frame of 10 s was set to measure mass flow rate for each alginate hydrogel recorded at each pressure interval. Volumetric flow rate for both theoretical and experimental data was plotted as shown in Figure 3-7.

The model was obtained with the following assumptions

- i) No slip at wall
- ii) Fluid is incompressible
- iii) Flow is steady, laminar and time independent
- iv) Viscosity is not affected by pressure changes along the channel
- v) Entrance and exit effects are negligible

Figure 3-7 A Preliminary model set up to describe volume flow rate of hydrogel extruded.

The general trend displayed in the model is similar to the experimental data for both hydrogel concentration tested. However, there seems to be a shift between the model and the experimental results. The current predictive model sees an accuracy of 50% in general.

Another parameter for modelling is the width of the printed struts. Distance between needle and platform is held constant at $D = 0.1\text{mm}$. As cross-sectional area 12:1 and 12:2 alginate hydrogel printed on glass slide displayed more of an ellipse as compared to sphere, width of hydrogel strand is modelled with equation describing the area of a half-ellipse. As such flow model describing volumetric

flow rate of respective hydrogel was used to correlate platform's moving speed with width of hydrogel strand.

$$\frac{Q}{V_m} = A_E = \frac{\pi \times a \times b}{2} \quad \text{----- (11)}$$

Figure 3-8 Preliminary model relating width of hydrogel struts to flow rate of hydrogel extruded and moving speed of platform.

Both Figure 3-7 and Figure 3-8 shows that experimental data and model prediction follows similar trend. The accuracy of the predictive model increases with increasing speed. Experimental data obtained showed 59 – 84 % accuracy for 12.1 Alg model and 45 – 65 % accuracy for 12.2 Alg. For better estimation, more factors can be considered for calculation. For instance, other studies have

used Herschel-Bulkley model that includes yield value of colloidal gel for estimation of flow rate [187]. Another factor that can be considered is the fluid's velocity due to wall slip [187, 225]. Li's team reported a better estimation by considering wall slip factor. Main difference between present hydrogel study with Li's is that colloidal gels comprises of 30 μ m hydroxyapatite particles dispersed in chitosan solution was used. As such, influence of wall slip condition on flow model may require further studies for alginate hydrogel flow. Further analysis is required in terms of determining particulate size that may be present in hydrogel.

Another explanation for the deviation could be the estimated value of alginate's density. In the current setup, density of alginate is assumed to be close to density of water ($\rho = 999.97 \text{ kg/m}^3$). More sophisticated measurements can be made to obtain the hydrogel's density using acoustophoresis [226]. Additional factors can be considered in the width prediction model for better approximation. Firstly, height difference between needle and printing platform is set at constant, $D=0.1\text{mm}$. Hence, distance between needle and platform can be investigated. However, as stated by Wang, path height does not have significant effect on width of construct [221].

Through this investigation, producing hydrogel strands with spherical cross-sectional area may require either increasing the surface tension energy of hydrogel or reducing the interfacial energy between hydrogel and contact surface.

An approach is to increase viscosity of hydrogel through increasing concentration. However, increasing concentration will inevitably increase the minimum pressure required to produce continuous hydrogel strands as demonstrated in this experiment. Another possibility is to reduce the interfacial energy through printing in solution. The buoyancy effect from the solution provides an upward force that reduces the interaction between hydrogel deposited and print surface.

Another approach that can produce hydrogel strands with spherical cross section is to crosslink hydrogel beyond sol-gel transition immediately. UV curing of hydrogel strand can be used on photopolymer to arrest spherical sectional area shape of material upon exiting from syringe needle. Several photopolymers such as GelMA, and PEGDA have been employed to produce hydrogel construct with structural fidelity [150, 158]. Most reported photopolymer produces spherical hydrogel strands. Moreover, chemically crosslinked photopolymer shows better stability and shape fidelity as compared to hydrogel formed through physical crosslink.

Additionally, the preliminary model established in this entry hopes to provide a basis for future studies on mechanical forces (fluid shear) on microlevel organization in hydrogel. Several studies have identified the influence of strain on orientation of collagen fibre [227, 228]. Such external forces affect the internal organization of hydrogel and the effect of fluid shear in time-pressure extrusion

based system may be used as a mean to control the internal organization of hydrogel.

3.4. Summary

Cross-linked hydrogel was used to investigate the influence of printing parameters and material properties in extrusion-based bioprinting. The minimum pressure required to extrude material is dependent on the material's viscosity. As crosslinking density of hydrogel increases, a higher baseline pressure is needed to extrude hydrogel strands. Moreover, material-substrate interaction was shown to affect print width and can potentially be a factor for evaluating print resolution of hybrid bioprinting system. Non-linear relation is identified between the moving stage speed and hydrogel width extruded. Further investigations can be made to develop mathematical models that capture material-substrate interaction for better understanding on the effect of print resolution when multi-materials deposition is made. The ability to control print width through printing and material factors allows control over print resolution.

Several parameters that determine the width of dispensed hydrogel using a time-pressure system were investigated as described above. Power law model was established to estimate volumetric flow rate of hydrogel dispensed. Although experimental data showed similar trend towards the model, there seems to be some deviation. Subsequently, width of dispensed hydrogel was related to volumetric flow rate of hydrogel. Cross-sectional area of hydrogel dispensed was approximated as half ellipse. Further studies are required to identify additional factors that can attribute to shape fidelity of hydrogel strand. As with other

optimization studies, models presented in this chapter provides a platform for future effect-relation studies of time-pressure dispensing system.

Chapter 4. Material formulation and bioprinting of

C2C12 cells

4.1. Introduction

Formulation of material for bioprinting requires considerations in the aspects of cell/tissue requirements and process compatibility (Figure 4-1). The ideal material properties for bioprinting are biocompatible and with suitable degradation kinetics that matches the rate of ECM replacement from cells. Moreover, mechanical properties of construct should match the needs of intended tissue types. An optimized material yield favourable cellular response such as enhanced force production in engineered muscle tissue [229]. Also, printability of material is defined as the suitability of materials to be accurately and precisely deposited with desired spatial and temporal control [213]. Most importantly, the choice of material is influenced by the ability to protect cell viability during printing process. During bioprinting process, cells and materials are subjected to the mechanical stresses specific to the printing technology. Such process-induced mechanical forces may influence cellular response and the intracellular structures of the cell [230]. For instance, proliferation potential of human mesenchymal stem cells (hMSCs) was significantly lowered when cells experienced higher shear stress (>10 kPa) during printing; whereas the potency of hMSCs remains unaffected with higher shear stress [204]. As such, it is important to ensure that printed construct contains viable cells for maturation.

Shear stress is inevitable in any dispensing process [204]. However, the magnitude of shear stress in a time-pressure extrusion-based bioprinting system is controllable through varying printing parameters such as nozzle diameter and printing pressure [230]. It is a general consensus that in extrusion-based bioprinting, an increase in the pressure and reduction in nozzle size decreases cell survival primarily due to the increase in shear stress experienced by cell-material during extrusion process [213]. Nozzle diameters and printing pressures are two major printing parameters that have been investigated to influence cell viability, where a range of 40 – 99% cell survival rate has been reported [230, 231].

Hydrogel composites with dual crosslinking system, such as gelatin and alginate, have been commonly used in bioprinting [231-234]. Similarly, such combination can be found in chemically modified GelMA with alginate and is prevalent in many biological studies, not restricting to bioprinting [207, 235]. The presence of alginate enhances mechanical properties of the hybrid hydrogel while providing different crosslinking mechanism for the hybrid material. Sodium alginate upon exposure to calcium chloride results in a physically cross-linked construct that is reversible through chelation of the calcium ion. This serves as the primary crosslinking mechanism for the hybrid material. Acrylate group in GelMA provides the functional groups for secondary crosslinking through exposure to UV radiations. Such chemical crosslinking mechanism forms stable construct after printing, hence allowing maturation of engineered construct.

Viscosity of GelMA is affected by polymer concentration and the printing temperature. As the ambient temperature increases, the viscosity of GelMA reduces. Such thermal property has been used to improve the printability of GelMA. By reducing the printing temperature, GelMA at lower concentration (3-5% w/v) can be printed into concentric and cubic shapes with fidelity [174]. Most importantly, the acrylate group chemically added to gelatin polymer backbone allows permanent crosslinking of hydrogel to yield a stable hydrogel structure that can withstand cell culturing as opposed to pristine gelatin.

A range of cell viability (40 – 99%) for extruded cells in bioprinting has been reported across different research groups. This can be attributed to differences in i) material composition (e.g. type of hydrogel, type of cells, cell concentration) ii) bioprinting setup (e.g. ambient temperature, processing temperature) iii) cell viability test conducted. Hence, it is essential to establish a baseline for evaluating cell viability pertaining to the current fabrication setup. As such, a framework has been established and used to formulate material for extrusion-based bioprinting of C2C12 cells (Figure 4-1).

Figure 4-1 Framework in material selection process for bioprinting.

4.2. Materials and methods

4.2.1 Synthesis of Gelatin Methacryloyl (GelMA)

GelMA was synthesized by first dissolving 10% w/v Gelatin Type A (Porcine skin, Sigma-Aldrich, G2500) in 1X PBS (pH 7.2, Vivantis) at 60°C stirring at 600rpm for an hour. 1.4% v/v of Methacryl Anhydride (Sigma-Aldrich, 276685) was added dropwise and the reaction continued for 2 hrs at 50°C. 1X PBS was added to quench the reaction. The mixture was transferred into dialysis tubing (MWCO: 12400, Sigma-Aldrich) for dialysis in distilled water for 4 days at 40°C. Finally, the solution was lyophilized for 7 days, and stored at -20°C for future use.

4.2.2 Hydrogel preparation

Hydrogel with varying compositions were prepared as per Table 4-1 below. Firstly, alginate was added to 10mM HEPES buffer warmed at 37°C. The mixture was held at 37°C for 15 mins with occasional vortex prior to addition of GelMA. The composite material was held at 37°C for another 15 mins. Thereafter, Photoinitiator containing 10% w/v 2 – Hydroxy – 4' – (2 – hydroxyethoxy) – 2 – methylpropiophenone (Sigma-Aldrich) was dissolved in ethanol and added to the build material at 0.02% v/v

Table 4-1 Different material compositions for evaluation on biological and mechanical properties

	0% Alginate	2% Alginate
5% GelMA	5M0A	5M2A
10% GelMA	10M0A	10M2A
15% GelMA	15M0A	15M2A

4.2.3 Degradation studies

Hydrogel was casted into a 35 mm petri dish and cured under UV lamp for 120 s. A 5 mm biopsy punch is used to cut equal hydrogel pieces for degradation studies. Samples were submerged in 2.5U/mL of Collagenase Type II (clostridium histolyticum, Sigma) and incubated at 37°C. The degradation profile is evaluated at different time points (2, 4, 7 and 24 hrs).

4.2.4 Encapsulated cell proliferation studies

C2C12 (ATCC® CFL-1772™ Mus musculus muscle) rat myoblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM/High Glucose, Sigma) with 15% of Fetal Bovine Serum (FBS, Gibco®), 1% Antibiotics (HyClone™) and. The cells were maintained in an incubator at 37°C and 5% CO₂. Culture medium was changed every 3-4 days. Cells were subcultured and detached using enzyme, 0.25% Trypsin-EDTA (Gibco®) and centrifuged at 220g for 5 mins. Cell pellets obtained are counted and resuspended in appropriate concentration using hydrogel composites described in 4.2.2.

5E6 and 10E6 cells/mL of hydrogel were loaded into the 35 mm petri dish. Upon UV crosslinking, a 5 mm biopsy punch was used to cut out equal sized samples. The samples were cultured in 24 well plates. PrestoBlue™ Cell Viability Assay (Life Technologies™) was used to assess proliferation at different time point (Day 1, 3 and 5). Cell media was removed and washed thrice with 1X PBS. 500 µL of assay media was added into each well and incubated for 1 hour at 37°C. Assay media is a mixture of PrestoBlue® reagent: culture media with volumetric ratio of 1:9. Thereafter, 100 µL of the assay is removed and loaded into 96 well black plate. Fluorescence readings were conducted using Tecan Spark 10M (Excitation/Emission: 560/590 nm).

4.2.5 Rheological test

Mechanical properties of materials are characterized using parallel plate rheometry (Discovery Hybrid Rheometer, DHR2, TA Instruments). Uncured hydrogel samples were loaded onto the rheometer with gap size set at 550 µm. Oscillatory frequency sweep, 1% strain from 0.4 to 40 rad/s, was carried out under controlled temperature of 25°C with a soaked time of 180 seconds before results are recorded.

4.2.6 Bioink formulation and printing parameters

5E6 and 10E6 cells/mL loaded into 5 mL printing cartridge (Nordson EFD) containing the bioink 25G stainless steel needle (Nordson EFD, 7018333) was

attached to the printing cartridge and loaded into the bioprinter. The hybrid material containing cells were extruded using Biofactory® (RegenHU Ltd). Input pressure was varied according to the polymers' concentration of the hybrid material (Table 4-2). The cell-hydrogel mixture was extruded for a fixed amount of time (15 s) at various pressure settings to evaluate cell viability. To evaluate on the effect of UV exposure on the viability of cells, the cell-hydrogel extruded was cross-linked using the in-built UV pen ($\lambda = 365 \pm 10 \text{ nm}$, 3000 mW/cm^2) for 30 s.

Table 4-2 Variation in material composition and printing parameters for cell viability testing

Material (GelMA:Alginate:Cell)	Composition	Varying Pressure (Bar)
10M2A5C		0.5, 1, 1.5
10M2A10C		0.2, 0.5, 1, 1.5
15M2A10C		0.75, 1, 1.5

4.2.7 Viability test of bioprinted cell

Cell viability was using LIVE/DEAD® Viability/Cytotoxicity Kit (L3224, Life technologies) as per recommended protocol. Briefly, EthD-1 and Calcein AM stock solution was added to 10mL of sterile D-PBS solution to yield working solution with final concentration of 4 μ M and 2 μ M respectively. Cell viability was assessed through microscopic imaging (Axio Vert.A1, Carl Zeiss). Cells were counted using “Analyze Particles” from ImageJ. Percentage of cell viability was quantified as (No. of LIVE cells)/ (No. of LIVE cells + No. of DEAD cells) *100. Data were tabulated under various printing condition and evaluated. One tail t-test was used to assess the significance differences between data.

4.2.8 Microstructures of bioprinted hydrogel

To study the effect of bioprinting on microstructural changes in bioprinted hydrogel, a 15 mm cube was printed using GelMA/Alginate within a frame printed with 25% w/v Pluronic F127 dissolved in CaCl₂. To cure the bioink, the toolpath for UV pen (part of the Biofactory ® set up) was designed to follow the path of infill at 10 mm/min. After curing, the samples were coated with 1X PBS to remove from the glass substrate.

SEM sample preparation

Square printed samples underwent serial dehydration of 25, 50, 75, 90% ethanol in intervals of 15 minutes. The dehydrated samples further undergo critical point

drying (BAI-TEC Critical Point Dryer-030) and immediately gold coated (20mA, 60 s). Gold-coated samples were imaged using scanning electron microscope (JEOL JSM 5600-LV).

4.3. Results and Discussions

4.3.1 Degradation studies

2.5 U/mL of collagenase type II has been chosen to study the degradation trend across different material compositions. The concentration of collagenase selected was based on previous literature. Both lower (1 – 2.5 U/mL) [179, 236] and higher concentration of collagenase type II (25 U/mL) [237] have been used for GelMA degradation studies. The use of higher concentration of collagenase hasten degradation rate of GelMA hydrogel. In the study by Koshi, degradation study using 25 U/mL of collagenase type II was done in parallel with 10 µg/mL of recombinant mouse and human MMP-2. These results indicate that GelMA can be degraded with both collagenase and mammalian gelatinase. Matrix metalloproteinase (MMP) sensitive sequences in GelMA offer sites for enzymatic degradation. Material degradation is essential for engineered tissue constructs to allow cells to deposit newly formed ECM [236, 238]. Hence, it is important to evaluate degradation rate from different material composition. Moreover, with the addition of alginate in GelMA, the degradation profile is unknown. Thus, it is essential to assess the effect of polymer addition on hydrogel's degradation rate. Specifically, the influence of alginate and the effect of hydrogel swelling on degradation profile was investigated.

4.3.1.1 Influence of Alginate on degradation profile

The addition of alginate hydrogel affects the rate of degradation. As shown in Figure 4-2, the presence of alginate in the hydrogel composition sees an initial increase in the weight content across different concentration of GelMA (5, 10, 15% w/v). The degradation of 5/2/0 (12%) was slower than 5M0A (33%) at 0.5 hour. Similarly, in the degradation study on GelMA and modified hyaluronic acid methacryloyl (HAMA) mixture, the addition of second polymer, HAMA, decreases the degradation rate as compared to pristine GelMA [236]. HAMA, being non-reactive with collagenase, impaired degradation of the GelMA-HAMA mixture. Likewise, alginate used in the degradation study cannot be enzymatically degraded by collagenase. Hence, comparing X/2/0 and X/0/0 samples across different GelMA concentration showed a reduced degradation rate with samples containing alginate (X/2/0).

Figure 4-2 Mass change rate of different hydrogel compositions immersed in 2.5U/mL Collagenase Type II to investigate on the effect of polymer content in influencing degradation rate.

4.3.1.2 Hydrogel swells prior to degradation

In general, the swelling effect of 10 and 15% w/v GelMA stabilizes 2 hrs after incubation (Figure 4-2). Comparison at 2 and 4 hour intervals showed that percentage change in weight of the respective samples is larger in 10M0A (50% mass reduction) than in 15M0A (30% mass reduction). Higher macromer concentration increases mass swelling ratio [178]. High degree of crosslinking results in lower swelling ratio [239]. Diffusion of oxygen and nutrients is impaired due to the reduced pore sizes.

The current degradation study was conducted to simulate the state of hydrogel in bioprinted construct. It was observed that bioprinted constructs experience an initial swelling state after printing [240]. Similarly, it has been observed in the current study an initial swelling state within 0.5 hrs after incubation. Depending on the compositions, the degree of increment varies. The highest weight increment was observed in 15M2A samples with a total of 50% increase within 0.5 hrs of incubation. Whereas 5%w/v GelMA samples showed an immediate decrease in weight content after 0.5 hrs of incubation.

Swelling effect from hydrogel can be explained due to the degree of crosslinking in hydrogel. The degree of methacrylation (DA) of GelMA hydrogel affects the mass swelling ratio of the printed strand [178, 241]. DA is inversely related to mass swelling ratio, higher DA value resulting in an increase of acrylate for photocrosslinking. Hence, a higher crosslinked hydrogel resulted in lower swelling ratio. The swelling characteristics affect solute diffusion, mechanical properties, surface mobility and even surface properties of hydrogel strand [178]. Similarly, Audin *et al.* reported a sudden increase in GelMA struts upon culturing of the swollen struts [129]. GelMA struts swell approximately to twice its patterned width (from 50 μm to 100 μm) within 24 hrs of culture. Compensation for swelling is essential especially when bioprinting anatomically relevant structures [240].

Based on the evaluation on the degradation profile, bioink with 5%w/v GelMA were not selected due to its fast degradation rate. Prior studies have reported the use of low concentration GelMA (4 – 5% w/v) for C2C12 encapsulations [129, 242]. Comparatively, the reported GelMA displayed higher degree of substitution (50 and 80%). However, the GelMA synthesized in this experiment is predicted to have lower degree of substitution (DS), approximately 20%, based on the percentage of methacryl anhydride used [178]. Low DS GelMA is preferred in the case of extrusion-based bioprinting. It was shown that GelMA with low DS has higher viscosity even above its melting temperature [243]. Such property is essential in controlling printability of extrusion-based bioprinting at standard conditions [244].

4.3.2 Cell proliferation studies

From the previous section, both 10 and 15% GelMA were selected due to their stability during the degradation study. Hence, in this cell proliferation study, only composites containing alginate were evaluated. The reasons are twofold. Firstly, the presence of alginate in GelMA bioink forms interpenetrating network that enhances the mechanical properties of material [207, 235]. Matrix stiffness sensing has been demonstrated in various cell types, regulating different cell behaviour [245, 246]. Secondly, the use of dual crosslinking system is a strategy that can be used to enhance print fidelity of structure [244]. Hence, both 10M2A and 15M2A were further evaluated for cell proliferation.

To evaluate the cell proliferation profile of encapsulated C2C12 in the varying matrix composition, PrestoBlue® was used to detect cell viability at different time point of study. Viable cells reduce PrestoBlue® into red-fluorescent dye that can be detected through fluorescence measurements. From Figure 4-3, the viability profile of C2C12 encapsulated in 10M2A increases gradually throughout the proliferation study. Comparatively, fluorescence readings for cells that were encapsulated within 15M2A saw an initial increase while decreasing at day 5 of culture.

Figure 4-3 Cell proliferation study using PrestoBlue®.

An increase in macromer content in 15M2A samples resulted in stiffer hydrogel. The decrease in C2C12 viability on day 5 for 15M2A samples can be explained due to the increase in encapsulation stress for samples with higher macromer concentration. In general, loss in cell viability can be attributed to encapsulation stress, drying of material during processing, or stress due to transient swelling after placing samples in media [178]. As observed in Figure 4-2, 15M2A samples experienced a 50% increase in water uptake at 0.5 hrs. Although 10M2A samples also experienced swelling effect, the percentage change was lesser with a 29% increase in water uptake. Hence, 10M2A was used for subsequent printing experiments.

4.3.3 Rheological testing

As discussed previously, the addition of alginate was to enhance the mechanical properties of hydrogel while providing a second crosslinking mechanism as part of printing strategy for enhancing definition of printed structure. While controlling grid patterning in Figure 3-4, there was no obvious distinction between vertical and horizontal struts that had been printed at varying layers. Hence, the use of a dual crosslinking mechanism is essential for enhancing print fidelity [244].

Rheological test comparing both 10M0A and 10M2A shows that the addition of alginate improves viscosity and viscoelasticity of 10% w/v GelMA (Figure 4-4). Both storage and loss modulus of 10M2A was higher than 10M0A; similarly, the complex viscosity profile for the hydrogel composite with alginate was higher as compared to pristine GelMA. The use of additives improves printing process and shape fidelity because of increased viscosity [177]. Grid constructs that were printed using hyaluronic acid and GelMA showed distinct lattice structure as compared to using GelMA alone. Similarly, as shown in the addition of alginate in Matrigel, compressive modulus of the bioink enhances the compressive modulus, hence improving the printability of bioink [247]. This further justifies the use of alginate in the composite material for better extrudability.

Figure 4-4 Rheological properties of 10% GelMA composite samples A) Frequency sweep B) Complex viscosity.

4.3.4 Printing process

Systematic evaluation of potential materials with C2C12 without consideration the effect of extrusion-based bioprinting has been discussed previously. However, the effect of extrusion-based bioprinting is necessary to understand the influence of mechanical stresses on cell-material. Also, the effect of UV exposure on cell viability needs to be assessed with the use of photopolymer GelMA.

4.3.4.1 Effect of polymer and cell concentration on printing pressure

To evaluate on the effect of loading cell density on printing pressure, two loading densities were used, 5C (5E6 cells/mL) and 10C (10E6 cells/mL). The minimum pressure required to extrude materials 10M2A10C, 10M2A5C, and 15M2A10C increased from 0.2, 0.5 to 0.75 bar respectively. When a fixed duration of extrusion period was set (15 seconds), it was observed that the number of cells captured across different setting increases with increasing pressure (Figure 4-5). The number of cells extruded in 15M2A10C is lesser in all printing pressure settings as compared to 10M2A5C and 10M2A10C.

Figure 4-5 Fluorescence microscopy comparing extrusion of bioink with varying concentration for a time frame of 15 s across different pressure settings (white scale bar = 200 μ m; green and red denotes Live and Dead cells respectively). Higher cell death was observed in 15M2A10C as indicated by region bounded in dotted red.

With higher polymer concentration, bioink viscosity increases. The influence of polymer concentration in materials' viscosity was demonstrated previously in Section 3.3.1. Minimum pressure needed to extrude material is related to materials' viscosity. Similarly, comparing the viscosity between 10M2A10C and 15M2A10C, the minimum pressure required to extrude material increased from 0.5 to 0.75 bar. It is demonstrated that higher concentration of GelMA in 15M2A10C required a higher input pressure to overcome the materials' inertia. GelMA with lower polymer concentration (< 10% w/v) sees higher cell viability

(> 80%) [178]. Cell concentration of bioink influences that materials' viscosity. Higher cell concentration in 10M2A10C, as compared to 10M2A5C, showed a reduction in bioink viscosity as demonstrated by the lower minimum pressure required to extrude material. Previous studies have shown that higher cell concentration in bioink reduces bioink viscosity [150, 166, 204]. A higher initial cell density is preferred as a higher loading density can accelerate tissue maturation [166].

When higher viscosity material is printed, the chances of nozzle clogging arises [232]. This could explain the fluctuation of cell viability in 15M2A10C (Figure 4-6). The deviation in 15M2A10C could be attributed to inhomogeneous cell distribution when mixing in high viscosity material. Also, the lower cell viability demonstrated in 15M2A10C can be attributed to the increase in stiffness of matrix, as shown by Zhao *et al.* where cell viability is inversely proportional to the storage modulus of hydrogel mixture [231].

Figure 4-6 Graph comparison between cell viability between different bioink composition containing different loading cell density.

4.3.4.2 Effect of cell concentration on cell viability during extrusion

Bioink comprised of different cell concentration (5E6 cells/mL and 10E6 cells/mL respectively) were extruded. Statistical analysis was conducted to evaluate on the significance of cell concentration in influencing cell viability of extruded construct. In general, extruded cells showed more than 90% viability despite the exposure to shear stress.

Comparing cell viability between the two material compositions extruded at its lowest setting (0.2 Bar in 10M2A10C and 0.5 Bar in 10M2A5C), the decrease in cell viability is significant. Extruding cell-hydrogel with higher viscoelastic resulted in lower cell viability. In a study by Zhao et. al, higher viscoelastic

hydrogel, resulted from an increased in holding time, yielded significantly lowered cell survival rate immediately after extrusion [231]. The effect of shear stress on cell viability was insignificant within the current pressure range (Figure 4-7).

Figure 4-7 (A&B) Fluorescence microscopy of LIVE (green) and DEAD (red) cells respectively. (C&D) Data acquisition to quantify number of cells using ImageJ for LIVE and DEAD cells respectively. (E) Comparison between cell viability (* denotes significant difference at $\alpha=0.05$, ** at $\alpha=0.10$ using one tail t-test).

The current study investigated printing pressure within the range of 1 bar with 0.5 increments. Similarly, Fedorovich *et al.* showed that despite increasing printing pressure from 2 to 3 bar, the reduction in cell viability is insignificant [248]. When proliferation profile of extruded cell-hydrogel was compared 5 hrs after printing and 24 hrs in culture, it was found that these results do not differ from cell-hydrogel construct that have been casted.

Subjecting cells to adequate level of shear stress can be beneficial to the tissue maturation in terms of enhancing cell proliferation. When cells were subjected to

different level of shear stress (< 5kPa, 5 – 10 kPa, > 10 kPa) versus control group (0 kPa), it was found that rate of cell proliferation is higher when low and medium level of shear stress is applied as compared to control [204]. However, cells printed at high shear stress showed a significant decreased in the proliferation rate. These results may indicate that a moderate level of shear stress is needed to enhance cells proliferation. Similar studies have established that higher shear stress (> 150 kPa) induced lower cell viability at less than 50% [150]. This shows that moderate shearing can improve cell function.

4.3.4.3 Effect of UV radiation on cell viability

UV curing is performed using a UV pen with spot size estimated at 5 mm. The cell-hydrogel were exposed to 30 s of irradiation. Comparison between extruded cells in 15M2A10C at 3 bar, the difference in cell viability is insignificant (Figure 4-8). Most experiments demonstrated varying UV exposure time from 30 s to 30 mins and light intensity [248-250]. Lower irradiation dose of 1800 mJ/cm² yielded higher viable cells [150]. Minimizing contact with photoinitiator radicals help minimize deleterious effect on cell viability in UV exposed cells [249]. Increase in UV exposure time from 15s to 60s reduces cell viability from 70 to 40 % [251]. The increase in UV exposure yields stiffer construct [177]. Synergistically, an increase in the number of free radicals and stiffer construct due to increase crosslinking network, is detrimental to cells and attributed to

lower cell viability reported. Hence, it is essential that UV exposure on cells is minimized.

Figure 4-8 Comparison between cell viability of cells exposed to UV and as printed (NUV).

4.3.4.4 Effect of printing on microstructure of hydrogel

SEM images from Figure 4-9 demonstrated staircase effect in bioprinted hydrogel highlighted using red dotted lines. This effect is distinct both in the x, y and z directions. As the toolpath of the infill is designed to follow a zig-zag pattern, the rounded edge shown in C displayed the turning path of the pattern. Such features are prevalent in other additive manufacturing processes [252]. These intrinsic features indicate that the printing process induced structural differences, a characteristic not observed in casted hydrogel.

Figure 4-9 Schematic illustration of SEM sample preparation showing microstructures in the exposed region (A); (B&C) Lamination effect of printed hydrogel (red scale bar: 20 μm ; white scale bar: 200 μm).

Comparing microstructures at higher magnification, it was observed that extrusion process induced microstructural changes on the printed material as compared to casted hydrogel (Figure 4-10). Comparison between casted hydrogel of collagen with 10M2A, showed that both casted hydrogel showed disorganised fibrous structure; whereas bioprinted 10M2A in showed anisotropic microstructures in comparison with casted 10M2A.

Figure 4-10 SEM images showing microstructure of casted hydrogel A) 5 mg/mL Collagen B) GelMA with Alginate (10M2A) and (C&D) bioprinted 10M2A showing directional preference along different location (red scale bar: 20 μ m; blue dotted line in C & D denotes the side at which hydrogel is printed unto substrate of glass slide).

4.4. Summary

A material selection framework was established for material formulation based on i) degradation profile of matrix ii) cell proliferation in matrix iii) rheology of material iv) technology specific requirements. Bioink with various compositions have been extruded and cell viability was assessed. The increase in cell concentration yielded bioink with lower viscosity, where lesser pressure is required to extrude the bioink. Similar with several findings, cell viability of extruded bioink within pressure range of 1 bar demonstrated insignificant reduction in cell viability. In both setup using 10% w/v GelMA and 2% w/v Alginate, cell viability remains high (>90%) across various pressure settings. Comparatively, 15% w/v GelMA yielded variability in the number of cell deposited. Through initial validation of an array material formulation and assessing cell viability in an extrusion-based bioprinting setup, the material formulation 10M2A was used for subsequent experiments.

The effect of printing on material was also investigated. Microstructural changes in bioprinted hydrogel construct were highlighted. Anisotropic microstructure was displayed in bioprinted hydrogel showing structural changes in printed and casted GelMA structures. This section has demonstrated a framework for evaluation of new material.

Chapter 5. Cell alignment with 3D organization through support-assisted bioprinting

5.1. Introduction

Resolution control and shape fidelity of printed construct is vital in bioprinting [244]. The importance of print resolution and fidelity lies in the control over spatial distribution of cells and materials, for better mimicry of native biological tissue and organs. Different strategies have been introduced to achieve control of shape and resolution of printed cell-hydrogel construct. A widely implemented strategy is through improving viscoelasticity of bioink using a semi-crosslinked bioink mixture or adding thickening agent into the bioink [29, 168, 177, 186-191, 253]. As hydrogel, the main material used to formulate bioink, is made up of 90% water, there is limited control over shape fidelity of bioprinted cell-hydrogel constructs.

While print resolution has been widely studied and reported, the discussion on shape fidelity has been less defined. In brief, print resolution defines dimensional features in the x, y plane whereas shape fidelity defines resolution in the z plane. The definition of 3D shape fidelity should not be restricted towards the ability to stack materials into a thicker construct. An illustration on the concept of shape fidelity can be found in Figure 5-1. In the case of 3-layered grid printing, the intended print was designed for overlaying struts with direction 0°-90°. However, the hydrogel printed collapses and the distinction between each layer was lost.

As shown in the microscopic image, there is no definition in the z-layer [254]. A new strategy, termed support-assisted bioprinting, has shown potential for enhancing structural and shape fidelity in bioprinted constructs [29, 188, 195-197, 199, 200].

Figure 5-1 Schematic illustration to demonstrate the lack of shape fidelity demonstrated in a 3-layered grid construct using bioprinting. Microscopic images taken from [254].

Support-assisted bioprinting uses the unique capability of bioprinting in heterogeneous material deposition. The materials used in support-assisted bioprinting are described as build and support materials. Build material is defined as the main bioink that contributes to the final bioprinted model, whereas support material is the ink that provides a scaffolding structure to hold the build material in place. Build/support configuration is common in bioprinting construct with high shape fidelity [29, 188, 195-197, 199, 200]. Hydrogels with reversible

crosslinking mechanism such as gelatin, Pluronic F127, and agarose are commonly used as the support material for easy removal post printing.

The purpose of using support-assisted bioprinting is to provide structural integrity for build material prior to final cross-linking of bioprinted construct. The ability to control print resolution and shape fidelity allows fabrication of engineered construct that better mimics the native tissue. For instance, cellular alignment in native myocardium wall varies with different angles across different planes [19]. Such complex anisotropic microstructure influences biological and mechanical function in native tissues [116-119]. Hence, numerous techniques have been proposed to initiate mechanical cues for cell alignment. These techniques can be broadly categorized as stretch-induced alignment and constraining cell growth towards alignment using geometrical designs. Designing geometrical constraints includes either selectively patterning biochemical cues to constraint cellular growth or fabricating scaffolds with anisotropic geometries [133, 134]. Hence, it is postulated that the use of bioprinting to control print path will also influence geometry of printed hydrogel which will direct cell alignment resembling the native tissue.

Several techniques such as integrating multiscale printing technologies [175, 197, 255, 256] and designing print nozzles for fabricating thin (approximately 200 μm) bioprinted constructs [251, 257] have been used to engineer bioprinted constructs with cell alignment. These strategies are in accordance to the alignment

mechanism described previously (stretch-induced alignment and geometrical constraints respectively). However, these techniques either requires elaborative setup or have intrinsic problems with cell seeding. There is a need for a novel fabrication method to produce i) structurally defined construct and ii) controlled cell alignment that varies with each z plane. In this study, support-assisted bioprinting is used to control print resolution and enhance shape fidelity of bioprinted cell-hydrogel. With well-defined construct coupled with the effect of shearing, cell alignment can be achieved to mimic alignment across multiple levels.

5.2. Materials and methods

5.2.1 Bioink formulation

Bioink comprising of 10%w/v GelMA and 2%w/v alginate were dissolved in 10mM HEPES buffer. Photoinitiator containing 10%w/v 2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Sigma-Aldrich) dissolved in ethanol and added to the build material at 0.02% v/v. 10E6 cells/mL were loaded into the bioink and extruded using 25G needle. Support material comprising of 30%w/v Pluronic F127 and 1M calcium chloride were mixed in volume ratio of 3:1 and loaded into syringe cartridge with 21G needle.

5.2.2 Design of lattice construct

A time-pressure dispensing system (Biofactory®, RegenHU) loaded with 2 cartridges (support and build materials) was used. Support-assisted bioprinting was used to create defined 0°-90° grid structure to assess the shape fidelity of bioprinted grid. The process begins by printing support material (Pluronic F127) in a 0° direction followed by infilling the gaps with build material (10M2A10C) along the same direction. Thereafter, a second layer was overlaid at an angle of 90° beginning with support material deposition and subsequent printing of the build material. Thereafter, the printed grid was crosslinked using the UV pen with exposure speed of 10 mm/min. Lastly, the support material was removed by washing crosslinked construct with chilled sodium citrate/EDTA. Thereafter, the

construct was washed with 1XPBS thrice to ensure complete removal of support material and sodium citrate/EDTA (Figure 5-2).

Figure 5-2 Schematics of using support-assisted bioprinting process in using support material (Pluronic F127) with build material (bioink: GelMA and Alginate with cells). Pluronic F127 provides temporary support prior to UV curing of the bioink for enhanced stability in cell culturing.

5.2.3 Tissue culture of the printed cell-hydrogel

The printed and seeded constructs were supplemented with cell media (High glucose DMEM, 15% FBS, 1% PenStrep) and incubated in 37°C, 5% CO₂. Cell culture media was replenished every two to three days. For the acellular grid, the

printed construct was inserted in a 24 wells plate followed by depositing 10E6 cells/mL of C2C12 cell suspension.

5.2.4 Immunofluorescence microscopy

Cells were fixed and fluorescently stained for F-actin (ActinGreen™ 488 ReadyProbes™, Life Technologies) and cell nucleus (NucBlue™ Live ReadyProbes™ Reagent, Life Technologies) according to the protocol. Firstly, the bioprinted constructs were fixed using 4% paraformaldehyde for 15 mins. 0.1% Triton X-100 in PBS was used to permeabilized the constructs for 15 mins, followed by washing with 1X PBS thrice. Thereafter, 3% bovine serum albumin in PBS was used as the blocking buffer. The samples were incubated for an hour at room temperature. The blocking buffer was removed, and samples were washed thrice with 1X PBS. Two drops of ActinGreen™ 488 ReadyProbes® and NucBlue™ were added to each printed sample. The constructs were incubated for thirty minutes away from light. Thereafter, the staining solution was removed, and the samples were washed with 1X PBS for three times. Fluorescence imaging were conducted using both Vert.A1 and Zeiss LSM 710.

5.2.5 Quantification of cell alignment

Brightfield microscopic images were obtained using Carl Zeiss Inverted Axio Vert.A1 microscope. Quantification of cell alignment was performed using FibrilTool plug-in in ImageJ for quantifying fibrillar structures [258]. Data was

reported in a radial plot to demonstrate the distribution of cell alignment angle. Alternatively, Fast Fourier Transform (FFT) was also performed and quantified using Oval Profile plug-in for ImageJ [259].

5.2.6 Statistical analysis

Dimensional measurements were taken from microscopic images and measured using ImageJ. Normality of data was assessed using Chi-test. Differences between groups were analysed using non-parametric Kruskal-Wallis test ($p < 0.05$, considered to be statistically significant).

5.3. Results and discussions

5.3.1 Characterization for support-assisted bioprinting

5.3.1.1 Support material printing influence geometry for build material

The side profile of support material was evaluated to determine the height increment for printing of subsequent layers. As shown in Figure 5-3, cross-sectional profile of printed Pluronic F127 is found to resemble a semi-circular shape as opposed to a cylindrical morphology used in previous models in predicting the hydrogel printed width. With increasing printing pressure, the width and height of support material increased. However, it is notable that the shape of the cross-sectional profile does not vary with increasing pressure.

While keeping the printing speed of Pluronic F127 constant at 600 mm/min, the side profile of the support material was characterized at various pressure settings. As the printing pressure increases from 2.5 to 4.5 bar, the width and height of support material increased by 91% and 83% respectively. The increment follows a non-linear relation, similar to the trend described in Figure 3-7. As described in the simulated model in Figure 3-7, an increase in the printing pressure resulted in a non-linear increase in the volumetric flow of material. Although the model material in Figure 3-7 differs from the current study, both alginate and Pluronic F127 are Non-Newtonian fluids that demonstrates shear thinning behaviours.

Hence, the increment in both width and height of Pluronic F127 was shown to increase drastically with increasing printing pressure.

Figure 5-3 Cross sectional profile of support material (Pluronic F127) deposited on a glass slide with varying applied pressure. Support material was printed via a 21G needle, at constant printing speed of 600 mm/min. Distance between support struts were kept at 1mm.

Pluronic F127 was used as the support material that provides the mechanical integrity for the build material to be printed. As shown in Figure 5-4, the toolpath for build material was designed to infill the gap between two struts of support materials. The ability to control support structure printing enables the formation of desired width of GelMA hydrogel through managing the offset between support struts. With the use of support-assisted bioprinting, build material with low viscosity can be printed with controlled resolution by altering the offset between supporting hydrogel struts.

Figure 5-4 Schematic diagram to illustrate support-assisted bioprinting of build material (red) between support material (grey).

5.3.1.2 0°-90° grid shows definition in z-plane using support-assisted bioprinting

To show the effectiveness in enhancing shape fidelity using support-assisted technique, surface profiling was used to assess the contours of the 0°-90° patterns. In direct bioprinting setup, 0° struts were first printed followed by 90° struts. The increment in the height for second layer (90° struts) was set at 300 µm according to the dimensions obtained in Figure 5-3 (3.5 Bar, 600 mm/min). This value was used as the height increment for both direct bioprinting and support-assisted bioprinting.

As demonstrated in the surface profile of the grid pattern in Figure 5-5, the variation in the height of 0° versus 90° is distinct in construct printed using support-assisted bioprinting. In the isometric view of Figure 5-5, the color-coded profile indicates the height differences of the struts. The collapse of 0°-90° layers is evident in Figure 5-5, where both 0° and 90° struts showed no differences in height. The fusion of multi-layers has been previously reported (Figure 3-4 and Figure 5-1). The lack of print definition is evident regardless of the material used. However, the use of Pluronic F127 as the support material provides temporary guidance that allows second layer to be printed on top of the first, thus preventing the top layer from collapsing.

Figure 5-5 Comparison between the surface profile of 0°-90° struts printed with support-assisted bioprinting and direct bioprinting of build material (without support). Confocal microscopy of samples taken using Keyence VK-X210. Distinct differences between first and second layer is observed in support-assisted bioprinting; whereas material accumulation (circled in pink) is observed in direct bioprinting.

Another distinctive feature that showed the lack of shape fidelity in direct bioprinting is the fusion of nodal point at the junction where 0°-90° struts crosses (intense red regions). It is evident that there is an increase in the height at nodal points (orange area) whereas struts were uniformly coded in green with no distinction in the height differences between 0°-90° struts printed using support-assisted bioprinting strategy.

5.3.1.3 Enhance print resolution with support-assisted bioprinting

The influence of support material in controlling dimension of build material was assessed through measurements of build material's width at fixed settings (10M2A10C, 25G needle, Pressure = 1.2 Bar, Printing speed = 900 mm/min). With the use of support-assisted bioprinting, higher resolution was achieved as illustrated in Figure 5-6. An approximated 50% reduction in hydrogel width can be observed in direct bioprinting without support and support-assisted printing. Moreover, the deviation of hydrogel width in direct bioprinting is greater than support-assisted bioprinting. The importance of controlled resolution and shape fidelity is essential in bioprinting [244]. Controlling shape fidelity in bioprinting refers to the ability to create constructs with defined features in x, y, z orientations as per designed. As shown in Figure 5-5, the definition in z was shown with support-assisted bioprinting when 0°-90° struts existed at different plane.

Support-assisted bioprinting utilizes the capability for heterogeneous material deposition in bioprinting to improve shape fidelity of engineered tissue. From previous study relating printing parameters to print resolution, it was found that material-substrate interaction affects the cross-sectional geometry of extruded material [191]. A change in the printed substrate affects the dimension of hydrogel deposited, inducing changes regarding print resolution. Lowering surface tension between hydrogel and substrate resulted in smaller contact angle influencing dimensions of hydrogel [166]. Hence, as shown in Figure 5-6, the

width of build material using support-assisted bioprinting more consistent with smaller deviation while the width has been significantly reduced.

Figure 5-6 Comparison between direct bioprinting without support and support-assisted bioprinting on the struts dimension (*asterisks shows the statistical significance between groups at $\alpha=0.05$). Direct bioprinting without support produces thicker hydrogel strut width with higher deviation; whereas support-assisted bioprinting produces finer resolution and lower deviation with distinct z-layer differences.

5.3.1.4 Principles of support-assisted bioprinting in enhancing resolution and shape fidelity of printed construct

In support-assisted bioprinting, the support material is used to control width of lesser viscosity material by tuning the distance between the support strut. As such, the mechanical integrity of support material must be robust enough to provide temporary support while subsequent support removal is needed for creating porous bioprinted construct for further tissue maturation. Hence, it is important to ensure that the two materials are stable prior to final crosslinking of bioprinted construct.

Pluronic F127, a block-copolymer comprising of poly (propylene oxide) (PPO) with both ends capped with poly (ethylene oxide) (PEO) blocks, is a thermosensitive polymer where it exists as a solution at low temperature and a physical soft gel above the lowest critical solution temperature. Pluronic F127 is essential for extrusion printing as it is a shear thinning fluid with good shear recovery [173, 260, 261]. In an investigation on the characterization of Pluronic F127, 25% w/v Pluronic F127 displayed suitable viscoelastic properties for extrusion printing [260]. As demonstrated in Figure 5-3, the highly viscous support material displays sufficient mechanical integrity while providing buoyancy force for second layer deposition of lower viscosity bioink.

Balancing the hydrogel-in-hydrogel effect between complementary pair of F127 with gelatin is primarily governed by the buoyancy compensation effect.

Fundamentally, F127 and gelatin demonstrate distinct phase separation phenomena under normal conditions, attributed to the different hydrophile-lipophile interaction between the two material [262]. To induce homogeneous mixing of F127 and gelatin, addition of surfactant is required to prevent phase separation of the two gels. Alternatively, gelatin can be grafted onto carboxylated F127 to produce a homogeneous composite [263]. Comparatively, a homogeneous composite of gelatin and F127 can be achieved through mixing cold F127 (4°C) with heated gelatin (50°C) [264]. Due to the immiscibility of both materials under standard operating conditions (pristine F127 with gelatin/GelMA, 25°C), this complementary pair of build/support material was used to enhance print fidelity and resolution of bioprinted construct.

5.3.2 Cell alignment in bioprinted grid structure

5.3.2.1 Homogeneous cell distribution in extrusion printing of cell-hydrogel

To investigate on the effect of printing on directing C2C12 alignment, bioink comprises of cell-hydrogel and hydrogel only were printed in 0-90° grid structure. Dimension of acellular struts printed were $467 \pm 39 \mu\text{m}$ as compared to cell-containing struts of $444 \pm 82 \mu\text{m}$. As shown in Figure 5-7, there were differences in the homogeneity of cell distribution between the two setups.

Figure 5-7 Comparison between cells seeded on scaffolds versus cells printed within the hydrogel. (red scale bar: 200 μm).

Cells that were printed as cell-hydrogel bioink are homogeneously distributed across different struts (Figure 5-7); comparatively, cells can be observed to cluster around the interstrut regions of the seeded scaffold. The agglomeration of cells at Day 2 can be observed whereas the size of aggregates was estimated at 300 μm (Figure 5-8).

Inhomogeneous cell distribution is an intrinsic limitation in manual seeding. Cells grow into spheroid-like cluster when manually seeded onto a hydrogel surface [265]. Such clustered growth has significant impact on cell-cell interaction. Moreover, due to the diffusion limit at 100 μm , the efficiency of nutrient and oxygen is limited with increasing cluster growth [265, 266].

Figure 5-8 Growing of cell cluster observed in Day 2 of culture. Red dotted circle indicates area of clustered cells due to inhomogeneous cell seeding in Day 0.

Despite the occurrence of cell aggregates growth, cell proliferation and elongation were observed along the struts at day 2 of culture. Approximately 42% of cells were aligned to the direction of printed strut. Cells are considered aligned when the angle calculated is within 15° . Upon culturing of the cell seeded construct at day 4 (Figure 5-9), the percentage of cells showing elongation increased to 57%.

However, while varying vertical plane (Plane A versus Plane B), the orientation of cell alignment varied with a 90° rotation within the blue dotted region (Figure

5-9). Cells growing at the Plane A demonstrated high degree of alignment within 3° along the strut. Comparatively, as the depth of view increased to Plane B, cell alignment within the blue region aligned along the vertical axis. This can be observed at various inter-strut positions.

Figure 5-9 Cell alignment profile of seeded cells at day 4. Blue dotted regions identified the change in cell alignment at varying z plane. (red scale bar: 100 μm).

The homogeneity in cell distribution within printed cell-hydrogel construct, indicated by the lack of cell cluster, renders bioprinting a value-added processing

technology for fabricating engineered tissue with homogeneously distributed cells. However, it is noteworthy that cell seeding of bioprinted construct is a convenient technique that enables co-culturing of different cell types for engineering tissue models with enhanced biological functions [267]. HUVECs cells were firstly mixed in a GelMA, alginate composite, followed by printing into a 0-90° lattice structure using coaxial nozzle with CaCl₂ fed as the fluid in the sheath flow. Neonatal rat cardiomyocytes were seeded onto the scaffolds and cultured for 15 days.

Such fabrication process of cell seeding has a limit on the number of cell types that can be incorporated within an engineered tissue. However, it is noteworthy that several microfluidic channels can be designed at the nozzle to incorporate extrusion of different cell types. As demonstrated by Kang *et al.*, the material composition and topography of the microfibres can be tuned through integrating six channels within the microfluidic platform [268]. However, such platform has yet to be integrated into a bioprinting system, where the main limitation lies in the short processing window of current materials due to the increased in complexity over control of multiple channels. Hence, by loading multiple print cartridges with cell-laden bioink has a better control over the organization and distribution of cells that can be printed within an engineered tissue.

5.3.2.2 Increased degree of cell alignment over culture

A timepoint study was conducted to understand the cellular behaviour of bioprinted construct. as observed in Figure 5-10, cell elongation can be observed during Day 3 of incubation. At day 3 of culture, majority of the cells remained circular, with a few cells displaying elongated morphology. As indicated by the FFT plot, the spectrum and peaks were not distinguishable indicating that there was no alignment at day 3. Group of cells that demonstrated elongated morphology increased on Day 4. As shown in Figure 5-10, a cluster of cells demonstrated elongated morphology spanning approximately 500 μm at day 4. As cells proliferate, the interaction of cells at struts level can be observed. Upon 14 days culture, cells proliferated and aligned across the entire bioprinted construct. Differences in the shape of FFT alignment plot in Figure 5-10 showed the increasing degree of alignment across different time point. Comparatively, the FFT plot for day 14 showed distinct peaks at 90° and 270° . This shows that more alignment is observed at day 14 of culture.

As compared to seeded construct, cell-hydrogel that was printed showed a slower rate of cell proliferation and cell elongation. A large population of cells demonstrated an elongated morphology at day 2 of culture (Figure 5-8) whereas similar degree of spreading was only observed in day 11 of printed construct (Figure 5-10). This can be attributed by the difference in the processing method (seeded versus printed).

Figure 5-10 Timepoint study of cell alignment in bioprinted cell-hydrogel struts across 14 days. Red arrows and regions indicate the elongation of cells.

Despite prior studies using FibrilTool plugin to assess structural alignment both in brightfield and fluorescence microscopy, the use of such image processing tool was not conventional in evaluating cell-based alignment. Conventionally, actin filaments of muscle cells are immunologically stained to assess and quantify the degree of alignment as shown in Figure 5-11. However, the use of image

processing software FibrilTool assessed the degree of alignment based on the shape morphology of cells. Both grayscale and RGB images have been analysed with FibrilTool and gave similar result [258]. Hence, degree of cell alignment can be assessed even with brightfield images. The degree of alignment reported based on assessing cell alignment with regards to its internal structure and cell shape/morphology should not differ as the cytoskeleton. Specifically, F-actin is a key determinant of cell shape [269]. Hence, the assumption to use cell shape as a form of measuring cell alignment is valid.

Figure 5-11 Confocal fluorescence microscopic images showing F-actin (green) and DAPI (blue) of the printed hydrogel struts (white scale bar = 50 μm).

5.3.2.3 Influence of print path affects degree of cell alignment in a two-layer bioprinted grid structure

Three locations that spanned across the printed lattice structure were identified to investigate on the effect of print path on cell alignment. The areas were labelled as A) perimeter, B) Turning angle and C) Interstrut. Degree and orientation of alignment differs across the different regions. FFT alignment plot on the

perimeter of lattice structure (Area A, Figure 5-12) showed broad and irregular spectrum with noticeable peaks at 90° and 270° .

Figure 5-12 Cell alignment profile at the perimeter (Area A).

The broad spectrum indicates that there was low degree of alignment. A similar setup was report by Aubin *et al.*, where C2C12-GelMA was photomasked into vertical struts with surrounding frames [129]. Cells along the perimeter showed random alignment. Comparatively, cell alignment along the perimeter of the construct shows small degree of alignment along the peripheral region. Distinct peaks at 90° and 270° indicated that the cells had preferential alignment along the horizontal direction (Figure 5-12). An explanation could be due to the direction

of printed toolpath, as indicated by the yellow dotted print path. The alignment in the horizontal direction is in parallel with the direction of extrusion print head when extruding cell-hydrogel.

When assessing cell alignment at the turning angle (Figure 5-13), there were distinct differences between the region along the peripheral (P) and cells that were deposited along the struts (S). From the FFT plot, the graph showed distortions with no obvious peaks at region P, indicating the lack of cell alignment within this region. Comparatively, cells that were deposited as struts in area S showed high degree of cell alignment with distinct peaks at 90° and 270° . In comparison, the peaks shown in Figure 5-13 have a narrower spectrum as compared to Figure 5-12. This indicates that cells laid within the strut showed alignment in parallel with the longitudinal axis of printed strut. An explanation for this difference lies in the width of hydrogel deposited along the perimeter versus the width of the strut. The average diameter of printed strut is approximately $380\text{ }\mu\text{m}$. whereas the width of hydrogel deposited around the turning angle is approximated at $1200\text{ }\mu\text{m}$, three times larger than the strut size. Hence, the degree of alignment is lesser at the area of turning angle.

Figure 5-13 Cell alignment profile at the turning angle (Area B).

Despite the weak alignment presented around perimeter of the lattice structure, degree of alignment within the strut (Area C) is high and directed along the longitudinal axis of print path (Figure 5-14).

Figure 5-14 Cell alignment profile at the interstrut (Area C).

The cell alignment within struts is determined by the direction of print, as indicated by the differences between horizontal and vertical struts (Figure 5-14). FFT alignment plot can distinguish between a horizontally and vertically aligned strut based on the difference in the peak patterning of the plot. The FFT plot for horizontal alignment along the x-axis is indicated by 90° and 270° peaks, whereas vertical alignment along the y axis of image is distinguishable with the 180° peak.

Comparing cell alignment along printed struts (Figure 5-14) with those along the perimeter of the printed grid (Figure 5-12), the degree of alignment (indicated by FFT alignment plot) is random in the perimeter as compared to those along struts. To determine the angle of orientation across the vertical and horizontal struts, FibrilTool is used to calculate the angular orientation of cells at different strut orientation (Figure 5-16).

The degree of alignment along the longitudinal axis of strut is high based on the rose plot presented in Figure 5-15, showing the frequency distribution of cells showing alignment within 15° of orientation. Approximately 80% of cells are aligned within the longitudinal axis of printed strut.

Figure 5-15 Cell alignment analysis over short range of 1 mm (scale bar: 100 μ m).

The transition between horizontal and vertical struts with aligned cells can be observed in Figure 5-16. The assessment of transitional changes is conducted through varying the microscopic images captured at varying plane within the same location. As the images were captured at fixed x, y position, cell alignment at 8 regions were used to assess the alignment changes across both lateral direction and height differences. The angle indicated is with respect to the x-axis of image. Both -90° and 90° indicates alignment in the y-axis, where negative values denote the direction of cell angle assessment, generated by the FibrilTool plugin.

Figure 5-16 Cell orientation over the interface between vertical and horizontal struts. Brightfield microscope image of cell alignment viewing from bottom layer of 0° struts, upper layer of 90° struts and the transitioning phase between 0°-90° struts. Angle of cell alignment is quantified and mapped along various position of the struts.

Cell alignment along the horizontal struts lies within 15° of orientation. As the viewing plane transitioned towards the interface between 0° and 90° strut, a transitional change can be observed between position 3 and 4. Cell alignment described in position 5 – 8 of 90° layer lies within 70°-90° of alignment, in parallel to the y-axis of image.

To demonstrate the ability of bioprinting with different angular orientation, a 0°-30°-60° grid pattern was chosen to mimic the angular changes across transmural

wall of epicardium (Figure 5-17). The fibre orientation at the epicardial layer varied from 53.42° to 29.73° at the endocardial layer [19].

Figure 5-17 Brightfield and fluorescence microscopy images to demonstrate the printing of cells varied at different angle orientation (Green: F-actin; Blue: DAPI; Red scale bar: 200 μm).

Conventional fabrication methods do not capture such complexity in angle orientation as these methods are limited to uni-directional stimulation for cell alignment along a single direction. For instance, uniaxial/biaxial stretching can only fabricate engineered tissue with a single angle orientation with regards to the stretch direction. Similarly, confined compression of scaffolds can only induce directional microstructures perpendicular to the direction of compression

[270, 271]. However, these methods are not adaptable to form complex 3D structures unlike bioprinting.

5.3.2.4 Mechanism of dynamic cell-hydrogel remodelling towards cell alignment in bioprinted construct

Cell alignment in tissue engineering has been extensively researched with various methods proposed to induce alignment and can be categorized as i) stretch-induced and ii) geometrical constrains. The influence of geometric constrains overrules the impact from stretch-induced alignment. When encapsulated cells were analysed at three different planar sections, cells at surface did not demonstrate stretch-avoidance as previously expected from constructs undergoing cyclic straining [124]. Presence of fibrous collagen at core can explain the absence of stretch-avoidance for cells that are embedded within the core where cellular dendritic extension into collagen fibrils within the core region.

A common fabrication strategy used to control geometric constraint is to reduce the width of cell-hydrogel encapsulates. Previous studies with encapsulated C2C12 at a concentration of 10×10^6 cells/mL using 5% w/v GelMA showed that the struts width of encapsulated cell-hydrogel affects the degree of alignment [129]. The findings concluded that struts with smaller width (50 and 100 μm) demonstrated higher degree of cell alignment. However, cell alignments were observed with current setup (struts size: 380 μm), showing the mechanism for cell alignment in bioprinted construct lies beyond inducing constrains or

imposing strain. In the current setup of using combinatorial effort of heterogeneous material printing and designing specific printing path, cell alignment with definition is achieved. The following describe the events that highlights key findings from previous sections to maps the mechanism of cell alignment in bioprinted constructs using support-assisted bioprinting.

5.3.2.4.1 Shear-induced microstructural changes in bioink

In this bioprinting setup, printed struts width was controlled at approximately 380 μm . With respect to the study conducted by Aubin *et al.*, such an increase in width of hydrogel strut will result in decreased percentage of cell alignment along the direction of strut [129]. However, the current setup shows cell alignment even with thicker struts size (380 μm), showing that the difference in fabrication method may induce different cell behaviour. A plausible explanation lies in the influence of extrusion-based bioprinting in inducing microstructural changes at material level. The directional control of material deposition using bioprinting (horizontal versus vertical struts) renders microstructural differences at varying planar level. The presence of anisotropic microstructure in bioprinted acellular construct was justified using SEM images in previous section (Figure 4-10). Such microstructural differences can provide geometrical and topological guidance to the encapsulated cells, hence inducing cell alignment at different strut orientation despite having a larger dimension of 380 μm . Similarly, shear force from bioprinting can potentially induce cell stretching [272]. With these results, it is

understood that shearing effect of extrusion induces changes both in cell and material aspect.

5.3.2.4.2 Migration of cells toward strut surface

During day 3 of culture, it is evident that most of the cells encapsulated within the strut demonstrated circular morphology (Figure 5-10). As indicated by the FFT plot, there was no distinct sign of cell alignment as shown by the lack of peak features in the plot for day 3. For cells that have been fully encapsulated in matrix, the process of cell spreading is a sequential event that is preceded by matrix degradation [273, 274]. Cells secrete matrix metalloproteinases (MMPs) resulting in a pericellular region comprising of viscoelastic fluid that enables cell migration [275]. The degradability of matrix affects the ability of encapsulated cells to spread. Hence, as indicated by the arrow of day 3 culture, a minority of cells were observed to be on the surface of strut.

Cell remodelling of hydrogel in gel compaction can be term as stretch-induced alignment, whereby the cell alignment was promoted through the presence of local tissue strains caused by gel compaction [127, 128].. However, gel compaction was not observed in current studies due to the difference in type of hydrogel used. The width of GelMA hydrogel struts did not decrease over culture. Similarly, 5% GelMA cell-laden struts fabricated by Audin did not report any reduction in strut size [129]. Due to the difference in hydrogel system between

GelMA and collagen, compaction was not observed in current bioprinted construct.

5.3.2.4.3 Initial cell spreading along strut surface

Cells showed extended elongation spanning from 600 – 900 μm as indicated in the red dotted region (Figure 5-18). Similar morphology of extended elongation of C2C12 cells was shown at day 4 of culture in Figure 5-10. Additionally, cells spread along the peripheral of hydrogel strut, identified by red arrows (Figure 5-18).

Figure 5-18 Cell spreading observed at peripheral of strut during day 4 of culture indicated by red arrows.

The preference for cells to migrate towards the peripheral of hydrogel struts can be explained as follows. Firstly, alginate presented in the bioink leaches out during cell culturing, hence eliciting larger pores for cell migration. Hence, the peripheral region of hydrogel struts resulted in pores due to removal of alginate.

This was previously reported by Zhang *et al.* where the alginate of the bioink composite can be leached out and dissolved in the culture medium in 5 – 10 days, resulting in larger pores along the borders [267]. Secondly, the preference for migration towards the outer region of struts can be explained due to the higher availability of oxygen and nutrients at the peripherals of the hydrogel strut [267, 276]. As such, the synergistic effect of increase porosity and better nutrients exchange at the peripheral attract cells migration towards the outer portion of strut.

5.3.2.4.4 Realignment along longitudinal axis of strut

Alignment of cells was first observed at the peripherals of hydrogel struts in Figure 5-10 and Figure 5-18. As understood from prior studies on inducing cell alignment in geometric constraints, cell alignment is an auto-catalytic process for myogenic self-organization [277]. Cells that are near the boundary tend to align first due to contact guidance. Subsequently, adjacent cells aligned according to the boundary cell. Such propagation of alignment information from the boundary cells is translated across cells through the autocatalytic feedback of rotational inertia. This self-organization of cells and propagation of alignment information from peripheral cells were also in the bioprinted struts. This was also observed in C2C12 seeded onto ridged surface of GelMA substrate [278].

With findings from previous sections, the mechanism for 3D cell alignment in bioprinting is proposed (Figure 5-19).

1 Shearing induced microstructural changes in extruded bioink

2 Encapsulated cells migrate towards strut surface

3 Initial cell spreading along strut surface

4 Realignment along longitudinal axis of strut

Figure 5-19 Mechanism of cell alignment in bioprinted strut.

5.4. Summary

In this section, the geometry of printed hydrogel (Pluronic F127) was characterized in the aspects of line width and cross-sectional area. Cross-sectional profile of support material is found to resemble a semi-circular shape as opposed to a cylindrical morphology. Measurement based on the width and height of support material deposited is essential for controlling build material's dimension through managing the offset between support struts. A 50% reduction in print resolution of build material is observed when comparing direct bioprinting with support-assisted bioprinting. Most importantly, construct with definition in z-plane (enhanced shape fidelity) has been shown. Differences in the viewing plane of 0°-90° struts shows that with support-assisted bioprinting, construct can be printed as per designed as opposed to planar stacking of hydrogel layers. Support-assisted bioprinting propose a technique where lower viscosity materials can be printed with shape fidelity. Moreover, this technique can be potentially incorporated with other cell deposition technology, not restrictive to bioprinting.

As such, support-assisted bioprinting was used to create 0°-90° grid structure for generating bioprinted construct with distinct cell alignment at different planar level. The grid patterning demonstrates the ability to control printing toolpath and illicit directional cell alignment – simulating the effect of angular changes in fibre alignment across native ventricular walls. A comparison study was conducted

between bioprinted cell-hydrogel construct versus cell-seeding of bioprinted acellular structure. Cell clustering was observed in seeded constructs, suggesting the inhomogeneity in cell distribution during manual seeding process. The clustering effect resulted in large cell aggregates ($>300\text{ }\mu\text{m}$) that may impair cell-cell interactions.

Timepoint study of the bioprinted construct suggested the onset of cell alignment during day 4 of culture. Hydrogel struts with approximate width of $380\text{ }\mu\text{m}$ were consistently fabricated. 80% of cell aligned within 20° of orientation. Angular changes between the interface of $0\text{-}90^\circ$ struts were characterized to show that cell strictly orientate according to the long axis of printed strut. Mechanism of cell alignment in bioprinted construct was established in this study.

Chapter 6. Enabling technologies for 3D bioprinting of the left ventricle wall

6.1. Data processing for freeform 3D bioprinting of the left ventricle wall

6.1.1. Introduction

Hydrogels, commonly used as vehicles for cell delivery in bioprinting, are high water content polymers with hydrophilic polymer chains that can be cross-linked to form three dimensional matrices [279]. Materials that are used for bioprinting are usually limited by their viscosity and gelling speed, hence reducing the process window to bioprint free-form biological structures [184]. Poor mechanical properties of hydrogels require development of bioprinting strategies to overcome such material-based limitations to bioprint freeform constructs [244]. As discussed in previous section, support materials are used to provide mechanical strength to hold the structure giving the overall form for the engineered construct. Cells and/or hydrogel (build materials) are subsequently printed to provide the functional components in a bioprinted construct.

The use of support-assisted configuration is prevalent in 3D printing especially in models with floating features and overhanging structures [280-282]. 3D printing software has the capability to automatically generate support structures needed for the build part, while some can assign different materials within a single build. Using materials of different properties, the supports can be removed

preferentially during post-processing [283]. The purpose for support structure in 3D printing is to provide structural integrity, allowing printing of successive layers when regions of object display overhanging or floating features. Support material is generated to partially or fully encapsulate the build part to prevent collapsing of overhanging and floating features [284, 285].

File preparation for bioprinting is one of the pre-printing processes that defines the structure of bioprinted construct. Many of the bioprinting setup uses similar data processing techniques as AM. To produce freeform structures with form resembling native structures, biomedical imaging data such as computerized tomography (CT) are used as the blueprint for generating bioprinter's toolpath [10]. Alternatively, CAD software such as SolidWorks can be used to design biological models for bioprinting [286]. Computer-assisted designs are sliced in layer-by-layer format (. STL or BITMAP images) and transferred in numerical control (NC) for bioprinting [150, 154, 287, 288]. However, processing print path layer-by-layer underutilizes the ability of coordinate based control over material deposition in bioprinting.

For instance, the native myocardium walls comprise of varying angle orientation that changes across different mural wall. For a left ventricular wall, the fibre orientation in the apical region varies from anterior to septum plane while transmurally there is a 30° variation across epicardial and endocardial layer (Figure 2-4) [19]. The printing toolpath can be programmed to follow the

curvatures of ventricle and the angular variation according to the degree of fibre orientation.

Deviating from the common 2.5D bioprinting of repetitive patterning to achieve increasing height, 3D bioprinting requires handling of larger data files that deliver coordinate specific information for freeform fabrication. As such, considerations in segmenting CAD files for bioprinting is also discussed in the following section.

6.1.2. Materials and methods

In general, files preparation for free form 3D bioprinting involves converting STL files into G-code that interfaces with the bioprinter. Overview of the novel approach as compared to conventional file segmentation approach is illustrated in Figure 6-1. RegenHU® Bioprinter with multi-printhead channels is used for the printing. A series of CAD segmentation method is used in the novel approach in preparing CAD files for bioprinting.

Figure 6-1 Process flow for file preparation for bioprinting, comparing between the two different file processing methods.

To assess functionality of the novel file segmentation approach, two models are demonstrated i) Free-Standing Coil (15mm) and ii) Left Ventricle Wall (Figure

6-2). Free-standing coil was used to initiate and develop the framework necessary for file preparation and segmentation of CAD models in bioprinting.

Figure 6-2 CAD models for printing of free-standing coil and left ventricle wall.

The schematics used for printing of free-standing coil using both the conventional and proposed file preparation framework for bioprinting of the free-standing coil is illustrated as follows (Figure 6-3). Pluronic F127 and GelMA/Alginate hydrogels were prepared as discussed in previous section as support and build and support materials respectively.

Figure 6-3 Schematic illustration of print process using conventional layer-by-layer printing versus file segmentation for biprinting of free-standing coil. Free-standing coil was used as the reference model for development of file preparation framework to print left ventricle wall.

6.1.3. Results and discussions

Generally, bioprinted models are converted into STL files which are uploaded to the bioprinter as G-codes. Comparing the two file processing methods, each file preparation method has its respective consideration factors as shown in Table 6-1. This method of segmenting files prior to printing has advantages such as i) improve structural stability of bioprinted construct ii) deposit multi-material iii) reduce overall printing time iv) overcome machine limitations. As conventional file processing deposits different materials according to each z layer, the printing process is shortened using proposed file segmentation. File segmentation shortens printing time through minimizing the need for interchange between printheads. Such printing approach first completes the print of a single material until critical parameters such as material instability and height limitation of printhead is reached before switching to the alternative material.

One distinct advantage of processing CAD models for bioprinting is the improvement on the design freedom of printed construct. The use of STL format for 3D printing requires deposition of material in a planar form, layer-by-layer. By using proposed segmentation method to print, materials can be divided into smaller fragments and will not be restricted by layer-by-layer printing. Table 6-1 summarises the consideration factors for file segmentation.

Table 6-1 Consideration factors for file segmentation

Consideration factors	Material's Compatibility	Machine's Compatibility
	<ul style="list-style-type: none">• Curing mechanism of material• Stability of material prior to curing• Interaction between different materials	<ul style="list-style-type: none">• Axis of movement• Height of platform• Distance between printed model and print head

6.1.3.1 Bioprinting of free-standing coil using file segmentation method

The coil is designed with interlacing of build and support materials, as shown in Figure 6-4. The segments in red and grey regions correspond to the build and support material respectively. As the coil has materials overlapping each other at multi points, the CAD model was sliced into different segments for fabrication (Figure 6-3). The prepared CAD file is then fabricated using both the conventional and new proposed file preparation methods. The fabricated coils are also shown in Figure 6-4.

Figure 6-4 Schematic illustration of print process using conventional layer-by-layer printing versus file segmentation for bioprinting.

In comparison, the time required for printing layer-by-layer using conventional file processing method is shorten. Moreover, shape of print is improved when printed in segments as indicated by the cylindricity of print.

6.1.3.2 Bioprinting of left ventricle wall using file segmentation method

A model of the heart's left ventricle is fabricated to show the capability of proposed new method for realistic bioprinting applications. Based on the file segmentation criteria, the current model does not require fragmenting the CAD model into smaller part files. CAD images of the structure fabricated, together with the generated support structure, is shown in Figure 6-5.

Figure 6-5 3D model and image of the left ventricle with build (red) and support (green) material. Curvature of printed left ventricle preserved after support material removal (curvature mapped with red dotted line).

The printed construct was cured under exposure of UV lamp prior to support material removal by washing with cold 1X PBS. The use of a support structure is essential in 3D printing. As shown in Figure 6-5C, the curvature of left ventricle printed was present even without the support structure. Printing of a thin hydrogel with curvature is possible using support materials as the shape of printed support provides the curvature for build material to conform to.

Using complementary hydrogel pairs as build and support materials has gained increasing interests [29, 188, 195-197]. While printed parts can be deposited into baths to print structurally robust constructs through buoyancy effect [199, 200]. the use of a support bath is disadvantageous mainly due to the wastage of materials. The ability to use multi-head printing allows controlled deposition of complementary hydrogel pairs, only printing the amount of material as needed.

6.1.3.3 Potential of 3D bioprinting for toolpath generation with angular orientation

Computer-assisted control of bioprinting allows coordination in multi-material deposition and direction of print path. The use of G-code allows coordinate control over the tool's direction, for potential use in printing tissue models that resembles anisotropic arrangement of cells and biomaterials in the three-dimensional space. As a proof-of-concept, the file for printing of left ventricle wall was further processed to produce G-code files that mimic the fibre and cell three-dimensional alignment in native myocardium (Figure 6-6).

Figure 6-6 Schematics for generation of print path with different angular orientation mimicking changes in the transmural left ventricular wall. Fibre orientation of native myocardium mapped out in [18, 19, 21, 22].

G-codes were processed at angles of 30° and 60° with curvature following that of the left ventricle wall. STL of left ventricular wall was obtained as described previously (Figure 6-6). Thereafter, equations for planes with 30° and 60° are generated. These angles correspond to an angular direction of fibre and cell alignment in native myocardium tissue [19]. The print path was simulated using CAMotics to visualize the G-code files. The codes were self-written to describe print path for angular 3D bioprinting with curvature that mimics fibre and cell alignment of native left ventricle wall. From the different view angles in Figure 6-6, it is apparent that the print path follows the curvature of the left ventricle wall resembling the native myocardium. This shows the feasibility of coordinate-based three dimension bioprinting.

6.1.4. Summary

Multi-material deposition is of increasing interest in bioprinting to improve print fidelity of 3D bioprinting. Specifically, the use of build and support materials have been demonstrated to build engineered tissue constructs with structural stability. The study on support material generation and designing print path is of great relevance for bioprinting as demonstrated across other additive manufacturing technologies.

In this section, the novelty in file segmentation for multi-material deposition in bioprinting has been demonstrated. This method is used to fully utilize the tool changing capabilities of bio printer for multi-material printing at a reduced print time. Considerations in terms of machine and materials compatibility are needed when adopting such file segmentation method across different bioprinter setup. Fragmenting CAD models and printing 3D bioprinted models into small section invokes novel bioprinting approaches towards printing structures that are more organic and closer to nature. Also, as a proof-of-concept, print path was generated to mimic the fibre orientation of native left ventricle walls. The angular changes across different ventricular wall was recaptured through varying x, y, and z coordinates in the print path.

6.2. 3D printed microfluidic platform for cell processing to form cell aggregates

6.2.1. Introduction

Two concerns remain in tissue engineering – engineering vascular network in engineered tissue and forming tissues with initial high cell density. As such, two areas of research in microflows manipulation can be observed in tissue engineering. One league of research identifies strategies to use microfluidic devices for processing cells in tissue engineering. These microfluidic devices can be formed through injection moulding, laser cutting or chemical etching [289].

However, such fabrication methods generally produce microfluidic chips with two-dimensional (2D) planar designs [290]. More intricate designs would lead to increased manufacturing complexity, production time and cost, for example, by employing methods such as direct projection on dry-film photoresist [291]. 3D systems fabricated using these techniques often require sophisticated designing, where a layer-by-layer structure is produced, and subsequent stacking and bonding are carried out to seal the components. Multilevel systems consisting of unconnected overlapping channels further increase the fabrication complexity, and techniques including the ‘membrane sandwich’ method [292] and the femtosecond laser direct writing [293] may be used for realizing such channels.

3D printing may serve as an alternative fabrication method due to its ability to produce complex and multi-level geometry. The fast fabrication of microchip in a single-step process without the need for manual assembly is a unique advantage of this manufacturing technique. The building process is automated with minimum human intervention, though steps are generally carried out for postprocessing of the printed pieces.

The printing of microchips was pioneered by McDonald *et al.* who investigated the printing of templates for PDMS casting using a solid-based printer [294]. The device has a minimum feature size of 250 μm . One of the first direct printing of microchip was accomplished by Moore *et al.*, whose work involves investigation of the behaviour of a valve channel in a microfluidic compact disk [295]. Recently, a cost effective printer based on the digital light processing technique was developed [296]. The capability of the printer was demonstrated by printing a few previously published microchips. The versatility of 3D printing has also spurred the development of auxiliary components, e.g. the 3D printed microchip interconnect with gasket and clipping mechanism, which was designed for addressing issues at the world-to-chip interface [297].

In this study, the aim is to evaluate on the properties of 3D printed parts for cell processing, specifically cell aggregate formation. As the materials used are commercial products developed by the 3D printers company, evaluation on the mechanical properties such as surface roughness and hydrophilicity of material

and biocompatibility of the materials are of utmost importance. Next, spheroid formation designs adapted from prior literatures are printed to evaluate on the effectiveness of 3D printed microfluidic to recapture the complexities of a 3D microfluidic device.

6.2.2. Materials and methods

6.2.2.1 3D printers

Two printers from Stratasys that use the polyjet and fused deposition modelling (FDM) techniques were tested. The polyjet printer used is the Object Eden350V with FullCure 720 and VeroClear model material with FullCure 705 support material. The completed parts were washed with water jet and subsequently immersed in ultrasonic bath (Sonorex Super) for 10 minutes for the removal of the support material. The FDM printer used is Dimension Elite with ABSplus-P430 as the model material and P400SR as the support material. The support materials were removed using the SCA-1200 support removal system, which involves the soaking of the printed parts in aqueous sodium hydroxide (NaOH).

6.2.2.2 Characterization of 3D printed materials

All physical measurements were performed at least five times for each test feature to achieve statistically significant results. For qualitative results, the scanning electron microscope (SEM) was used. Material characterization such as surface roughness and water contact angle were measured. Surface roughness was done using the Plμ confocal image profiler. The contact angle measurements were done using the Attension Theta optical tensiometer and the accompanied software from Biolin Scientific. 5 μL of distilled water was dispensed onto the printed platform for examination. Biocompatibility of the commercial materials was also assessed.

In brief, C2C12 cells were cultured in medium (Dulbecco's Modified Eagle Medium (Serana), 10% Fetal Bovine Serum, 1% Antibiotics) till 80% confluency. Cells were trypsinized using 0.25% Trypsin with EDTA, centrifuged, resuspended and counted before seeded onto printed samples (circular disc: 400 mm² x 2 mm) that have undergone post-processing conditions stated in Table 6-2. 0.7×10^6 cells were seeded onto the processed samples each to assess cell-material compatibility. The samples were incubated at 37°C overnight in a 5% CO₂ incubator and subsequently prepared for fluorescence staining using the Live/Dead Cell Double Staining Kit (Sigma-Aldrich®). Cell viability is assessed using fluorescence microscopy and quantified using ImageJ software.

Table 6-2 Sterilization condition of 3D printed material

Sterilization Condition	Duration
UV Exposure	1 hr.
70 % Ethanol	0.5 hr.
UV Exposure + 70 % Ethanol	1 hr. followed by 0.5 hr. respectively

6.2.3. Results and discussions

6.2.3.1. Surface roughness of printed features

As systems decrease in size, near-wall forces become the crucial factors in influencing flow. Surface roughness induced phenomena, for example, the van der Waals, electrostatic and steric forces emerge and were believed to offer explanations for observations that are unique to micro-scale flows [298]. In general, smooth surfaces are welcomed as they are more cell-friendly and are less prone to changing the intended flow dynamics. For example, shear stress was found to have negative impact by causing transient pores to form on cell membrane [299]. However, parameters associated with such cases are difficult to quantify, as several factors such as exposure time to stress, fluid viscosity and physiology of the cells act to complicate the process [184]. Comparing with the standard practice, untreated PDMS made using photolithographic casting was found to have an average roughness of 0.68 nm [300]. CO₂ laser cutting of polymethyl methacrylate (PMMA) microfluidic surfaces was able to yield a best roughness value of 100.86 nm with appropriate treatment [301].

The surface roughness value differs with the wall angle, channel curvature and channel direction, and varies significantly when different types of printers are used. As shown in Figure 6-7, the polyjet printer produces relatively smooth features.

Figure 6-7 SEM images of printed channels. Polyjet printed channel at A) 90° B) 30°; FDM printed channel at C) 90° D) 30°.

The surface roughness increases with increasing side wall angle due to the stitch marks formed. Results from the surface roughness test were plotted in Figure 6-8. From the trend shown in Figure 6-8, there were more irregular profile at 60° as compared to that at 15°. In fact, such trend is consistent for the spectrum for angles from 0° to 60°, which ranges from 0.47 to 8.44 μm . However, roughness at 90° decreases to about 1.7 μm . Singh has demonstrated similar range of surface roughness of polyjet printed material (0.68 μm) printed at 0° inclined angle [302].

In comparison, the FDM printer generates much rougher surfaces where the profile is characterized by a stepping feature. On the contrary to the trend shown

for polyjet printers, Ra values decrease at increasing wall angle, and range from 42.97 to 3.24 μm , whereas at 90° , there is a sudden jump in the roughness value to 12.6 μm . Referring to Figure 6-7C, the increase in Ra at 90° is due to the stacking layers of the printed materials. The Ra values of FDM printed parts was verified based on the definition of average Ra for each sloping angle θ at the preset layer thickness t of 178 μm .

Figure 6-8 Ra of the parts printed for various wall angles.

Besides straight channels, surface characteristics at different turning and slanting angles were visually examined. Referring to Figure 6-9, polyjet printer produces smoother surfaces. For channels at a slanting angle, the side walls printed by the polyjet printer are rougher than the straight channels.

Figure 6-9 SEM images of other channels. Round channels printed by A) Polyjet B) FDM; C) Slanting channel by polyjet yielded rougher wall.

Due to the novel nature of this technique, the printed parts' surface structuring and the effect on the performance of microfluidic devices is less studied. A concern is the impact on fluid flow and cell viability. Comparing with the Ra of less than 1 nm produced by photolithography using PDMS, both printers are much inferior with the use of the current model materials. However, the results do not serve to refute the feasibility of 3D printing as a candidate for biological microfluidic devices due to the following reasons.

Firstly, the amount of surface patterning and roughness desired depends on the application. For cases where smooth laminar flow is required, the presence of a rough surface could act as a hindrance. However, for devices that work on the induction of turbulence or convection, the surface features generated by the FDM printer will be highly welcomed. A case in point is the mixers, where complex flows are required to trigger chaotic conditions, as diffusion in laminar flow requires long channels and is highly inefficient.

Secondly, Ra values obtained in this study is not the actual values that will be used in microfluidic devices. Post-processing procedures such as surface

polishing can improve the surface condition. Paydar *et al.* was able to improve the surface roughness of PMMA-based printed parts by 50% with the use of wax [297], though more biologically compatible material is needed for tissue engineering uses.

Lastly, the role of surface patterning should not be overrated. For example, in a study that investigated the role of FDM printed capillary valve on the critical rotational frequency of a microfluidic compact disk, it was shown that though the device produced consistent offset from the theoretical prediction, the results followed the general trend closely and can be used as a functional device [295]. Though one cannot refute the impact of characteristic surface patterns produced by 3D printers on flow, proper calibration and careful studies may improve performance, depending on the application.

6.2.3.2. Water contact angle of printing material

In terms of the wetting ability, untreated PDMS is hydrophobic, which is usually not welcomed as it cannot be easily wetted by aqueous solution and is prone to the nucleation of bubbles within the channel [303]. Also, wetting at the fluid-wall interface determines the flow pattern. In particular, droplet formation devices produce water-in-oil droplets under hydrophobic surface condition, while oil-in-water droplets are produced in hydrophilic device [304]. Surface treatment of the fabricated systems can be performed to enhance surface hydrophobicity. Besides, surfactants of various concentrations can be added to the fluid phase to enact

changes to the solid surface's hydrophobicity. For example, when surfactants are added at larger than the critical micelle concentration, wetting property of the solid surface can be altered completely [305].

Figure 6-10 shows the results from water contact angle tests. FullCure 720 and ABSplus-P430 are hydrophilic, with average contact angles of 81.0° and 75.5° respectively. Another polyjet model material, VeroClear, was also tested and was found to be slightly hydrophobic, with an average contact angle of 93.0°.

Figure 6-10 Water contact angles of the model materials.

Water contact angle depends on several parameters, including material composition and surface roughness. In this study, only the contact angles on the horizontal planes printed with 0° inclination angle was measured. Also, drop size was known to influence the contact angle due to contact angle hysteresis [306, 307]. For example, another study which measured the contact angle of ABS at a number of drop volumes gave a value of 87.4°, which is 12° higher than that obtained here [295]. However, a detailed analysis of the contact angles is not the main aim of this study, which is to provide a rough estimate of the surface wetting

ability of the model material in comparison with that desired by microfluidic devices. Thus, only drop volume of 5 μL or 2.12 mm in diameter was used.

Since hydrophilic surface is preferred in most microfluidic channels, the presently tested model materials are desirable, though VeroClear is slightly hydrophobic. Though not investigated in this study, larger surface roughness was found to increase hydrophobicity [308]. Relating to the surface roughness results obtained, increasing the side wall angle may have an undesirable impact on surface properties. As roughness factor increases, roughness regime switches as air is trapped between the water droplet and the substrate surface. Large increase in water contact angle was observed by Yoshimitsu *et al.* [309], who studied surfaces with pillar-like structures with varying heights, at the onset of roughness factor increase, while contact angle stabilizes as roughness factor exceeds the threshold where a solid-air or liquid-air interface is formed. For the present case, since the model materials are not highly hydrophilic, rougher surfaces created by increasing wall angles should be avoided. However, just as in the case of surface roughness, wetting ability can be improved by post-processing, and results from these tests only serve as a guide for preliminary design purpose.

6.2.3.3. Biocompatibility of model material

The biocompatibility of the build materials is another essential prerequisite to assess the suitability of microfluidic chip for tissue engineering applications. Cell viability as a measure of biocompatibility of the model material was evaluated

quantitatively using a viability assay that is based on cell counting. Cell counting was performed after one day of culture in medium with the respective treated parts. Both model materials demonstrated good biocompatibility after subjecting to sterilization conditions stated in Table 6-2. The control set consisted of cells without any printed part. As shown in Figure 6-11, while ABSplus-P430 gives consistently high survival rate of above 95%, comparable with that of the control, across all sterilization conditions, FullCure 720 exhibited higher mortality rate of about 20% upon one hour of UV exposure. The fluorescence stained images for polyjet printed part under ethanol and UV treatments can be compared from images in Figure 6-12.

Figure 6-11 Cell viability under various treatment conditions.

Figure 6-12 Fluorescence staining of cells on FullCure 720 subjected to 70% ethanol and UV after overnight incubation.

The higher cell death after UV treatment could be due to the release of free radicals by FullCure 720, a photopolymer resin. While the constituents of the photopolymer are unknown, photopolymerization process is always activated by the presence of photoinitiators, which dissociate to form free radicals or ions upon radiation. In fact, this is a common concern associated with photopolymerization of hydrogel in tissue engineering applications, where photoinitiator concentration is a key parameter that compromises cell viability [310]. In this case, exposure to UV irradiation could have caused free radicals, in addition to those already present in the polymer, to be generated and remnants of

these interacted adversely with the cells, leading to detrimental cell death. Despite the difference, cell viabilities for all the treatment conditions are high, indicating the model materials are biocompatible.

6.2.3.4. Applications

There are three approaches for the fabrication of microfluidic devices by 3D printing, namely 1) direct printing of microchips, 2) direct printing of open channel microchip, which is bonded to top/bottom layers later, and 3) printing of masters from which microchips can be casted using conventional materials such as PDMS or PMMA. The first approach maximizes the advantage and flexibility offered by 3D printing and makes the fabrication process easier. The second approach is an intermediate method that facilitates the cleaning of support material, as removing by mechanical or chemical means is easier for an open channel. The last approach, despite being more cumbersome, is employed when the more established methods and materials are preferred.

In this study, both direct printing and the printing of open channels were performed, as difficulty in support material removal was expected. Of the two printers studied, the polyjet printer was chosen as the FDM printer uses an opaque model material, which makes flow visualization difficult, while the FullCure 720 used by the polyjet printer is more optically transparent.

6.2.3.4.1 Rotational flow device

The rotational flow device for generating tissue spheroids was adopted and printed [106, 107, 311]. A minimum channel dimension of 500 μm was used for a small percentage error of about 5%. As shown in Figure 6-13A, direct printing suffered from difficulty in removing the support materials, despite sonication and soaking of the part in NaOH. Only less than 2 cm of the materials were dissolved, with debris remained along the channel. In addition, swelling of the structure was observed after soaking in water for two days, as shown in Figure 6-13B. Alternatively, a three-layered design was adopted for indirect printing, as shown in Figure 6-14.

Unremoved support structure

Figure 6-13 Direct printing of rotational flow device. A) Channel blocked by support materials. B) Channel distorted by material swelling.

Figure 6-14 Assembly of the rotational flow device.

The open channel, though allow complete removal of support materials, has its own set of problems. The lack of knowledge of the model material led to difficulties in choosing an appropriate bonding method. Direct bonding using a colourless adhesive (Selleys Supa Glue) was performed, but this method produced inconsistent results as it depends largely on the skill of application of the adhesive. Specifically, over-application had led to channel blockage due to the sinking of the adhesive, as shown in Figure 6-15A. On the other hand, when insufficient adhesive was applied, leakage occurred, and this disrupts the flow dynamics. Figure 6-15B shows the outcome when flow was introduced through inlet of the bonded part. Negligible amount of the fluid flowed out from outlet A, as part of the channel was blocked by the adhesive. Unequal volumes of the liquid flowed to outlets B and C, as indicated by the different heights of the liquid columns, because of leakage. Consequently, evaluation of the device in generating rotational flow could not be carried out.

Figure 6-15 Printing of layered rotational flow device. A) Channel blocked by adhesive during bonding. B) Disturbed flow due to ineffective bonding.

6.2.3.4.2 Gradient Generator

The gradient generator was printed for examining the ability of 3D printer in fabricating multilevel channels. In comparison with the five-layered device

proposed in [291], 3D printing allows a minimum of two layers to be made, as shown in Figure 6-16.

Figure 6-16 Assembly of the gradient generator (Left) CAD design of gradient generator (Right) Printed gradient generator.

The use of fewer layers was enabled by sectionalizing the channels at locations along the channel, so that the sufficiently short channels allow the removal of the support structures by soaking in NaOH. Figure 6-17 shows a close-up view of the channel before and after cleaning, where the support structures had been completely removed. However, the device was not subjected to actual flow testing, since this study was unable to establish an effective bonding method.

Figure 6-17 Close up view of the gradient generator. Left) Before removal of support material. Right) After removal of support material.

Both the FDM and MJM/polyjet printers are unable to fulfil the high-resolution requirements of many microfluidic systems. The low accuracy level further raises the minimum channel size to 500 μm for 5% accuracy to be achieved. The presence of support structures and the lack of effective removal methods is a major engineering and design limitation that compromised the key advantage offered by 3D printing, which is flexibility and structural complexity.

However, one should remain cautiously optimistic about the use of 3D printing, as the problems highlighted are not inherent to the technology, but are rather a lack of appropriate materials. As the printers used in this study are not designed for microfluidic applications, they are not adept at fabricating at micrometre scale, but such shortcomings can be eliminated with more specialized machine design (e.g. smaller nozzle size and lateral and vertical displacement of the nozzle heads).

Furthermore, there are other printing technologies that are not explored by this study. A promising candidate may be the SLA, which is a liquid-based printer

that achieves better resolution due to the use of laser as the light source. Another option could be DLP printer, which also makes use of vat polymerization. Post-processing of parts printed by these printers involves flushing of the liquid resin, which might be achieved more easily than the cleaning of solid-based support structures. In addition, when the printer is configured for upside down printing of parts, the upward movement of the printing platform facilitates the flushing of liquid resin from the solid structure, and eases the cleaning process.

When considering the application of 3D printed microfluidics, several aspects are considered. Firstly, the dimension of fabricated microfluidic platform for biological application ranges from 100 to 1000 microns except for platforms that perform single cell analysis. Hence, most of the 3D printers can support such dimensional requirements. Moreover, the built materials must be biocompatible and support cells proliferation and survival. In most applications, microfluidic platform is subjected to incubation at 37°C for up to a week. Build material used must be mechanically and chemically stable under such conditions.

6.2.4 Summary

Two printers utilizing the polyjet and FDM technology respectively had been studied for their suitability of fabricating microfluidic platforms that are intended for tissue engineering applications. The printers were found to be unable to achieve the desired resolution and accuracy. Results from surface roughness and contact angle tests suggest that post-processing steps are needed for acquiring comparable surface conditions that are adopted by common microfluidic systems. The model materials used by the printers are biocompatible after subjecting them to sterilization processes, though polyjet materials may be averse to UV irradiation due to the release of radicals by the photopolymer. Complex post-processing steps are necessary for cleaning the printed parts, which poses many design and engineering limitations.

Despite the shortfalls, 3D printing offers the advantage of simplified process chain and the possibility for fabricating complex multilevel systems. While the printers that were investigated are not ready for making 3D microfluidic architectures, the concept is potentially viable and deserves further study. In fact, 3D printing technology that utilizes vat polymerization has proven to be a competitive candidate for the current purpose.

The current study used commercial printers though customized and self-build printers may be better options, as the available printers are not designed for printing at micrometre-scale. Future works should tap on the idea of 3D printing

and if possible, adopt a bottom-up approach, i.e. develop specialized printers for microfluidic applications, as commercial printers may not be compatible in terms of material availability, transparency, strength, etc. Another potential lies in the field of material development. In the current evaluated systems, build material used have been restricted by the ones provided by the manufacturers. With innovation in both printer's technology and material synthesis, 3D printed microfluidic for biological applications can generate culturing platforms rapidly in a cost-effective method.

Observations from this study seem to show that the current choice of printers and build materials are unsuitable for fabricating microfluidic chips. Several factors need to be addressed before implementing 3D printing techniques to microfluidic fabrication. These includes printer resolution, accuracy and the complexity associated with post-processing procedures, such as surface polishing and the cleaning of the printed parts.

Chapter 7. Conclusions and recommendations for future work

7.1. Conclusion

The research has fulfilled the main objective of this project: directed cell alignment using extrusion-based bioprinting for cardiac tissue engineering; devised a bioprinting strategy for improved print resolution and print fidelity; and identified enabling technologies for processing data and materials at pre-printing stage. The main contributions of this research are as follows:

- 1) A 0°-90° grid structure was printed with directed cell alignment along the longitudinal axis of struts. Cell alignment shown at varying layers has not been shown in the work found in bioprinted cardiac tissue. This is a major advancement in cardiac tissue engineering to mimick the variation in fibre and cell alignment across different transmural layers. In addition to the bioprinted construct with cell aligned, a mechanism at which bioprinted cells align was proposed.
- 2) A bioprinting strategy termed as support-assisted bioprinting was devised to improve print resolution and print fidelity of bioprinted construct. The knowledge derived from the previous print resolution studies showed the need to propose a bioprinting strategy to print with better definitions. Struts designed to orientate with varied angles at different z layers were well-defined when printed with support-assisted bioprinting. Improved

print definition through this strategy provided the infrastructure for cells to align distinctively at various z levels.

- 3) Two enabling technologies were identified and developed for pre-printing stages in bioprinting. This includes data processing for designing print path in multi-material 3D bioprinting and analysing 3D printed microfluidic platforms for processing cells. The print path designed was more biomimetic as it slice print models in angled planes resembling the architecture of native myocardium. The 3D printed microfluidic platform has the potential to produce chips with more complex design while the current studies provided information on the different considerations in designing and selecting 3D printed technology for microfluidic fabrication.

7.1.1 Directed cell alignment using extrusion-based bioprinting

A material formulation framework was also established to evaluate on suitable material composition for extrusion-based bioprinting of C2C12 cells. The framework provides a sequential evaluation of material for bioprinting of cells, not limiting on the technology and cells involved. This framework provides a quick evaluation for formulating materials bioprinting within a week. Bioink with the composition of 10M2A was used for subsequent experiments. In such setup, cell viability remains high (>90%) across various pressure settings.

Comparatively, a higher GelMA content yielded variability in the number of cell deposited. In this study, GelMA-alginate blend is studied.

Based on the material composition selected using the proposed framework, a 0°-90° grid structure was designed to mimic the differences in angle of fibre orientation of native left ventricle wall across the transmural wall. Cell alignment was observed along the longitudinal axis of 0°-90° grid structure. Cell alignment was evaluated using FFT plots and FibrilTool to quantify the degree of cell alignment through analysis of microscopic images. Cell alignment was observed in the current bioprinted constructs with hydrogel width approximated at 380 µm. From the current bioprinting setup, the presence of cell alignment at larger strut dimension (380 µm versus 100 µm) suggest an inherent difference prompted by the difference in other fabrication process reported (bioprinting versus hydrogel casting respectively). Moreover, in the current study to compare the degree of cell alignment using different fabrication method (bioprinting versus cell seeding), it was shown that cells in the bioprinted constructs were homogeneously distributed while cell alignment was directed along the longitudinal axis of hydrogel strut. Finally, the various findings were used to map the mechanism of cell alignment in bioprinting. The enhanced shape fidelity of 0°-90° grid fabricated using support-assisted bioprinting produced a dimensionally robust structure essential for creating cell alignment varied at different z layers. In the native myocardium tissue, fibre orientation varied across the transmural wall of left ventricle. Though previous research efforts have

emphasized on techniques to align cells anisotropically in engineered cardiac tissue, this is insufficient to recapture the variation of cell and fibre alignment in native tissue. The variation of cell alignment in the left ventricle wall provides both contraction and torsional motion for the heart. As shown in the current study, bioprinting was able to create cell alignment at varying angle across different z layers.

7.1.2 Bioprinting strategy for distinct 3D shape fidelity

A print resolution model was developed and described using a power law model to relate volumetric flow rate of hydrogel dispensed with print resolution. Several parameters such as printing pressure, stage speed, and material composition were identified to influence print resolution. The minimum pressure required for extrusion is influenced by the polymer's concentration and degree of crosslinking agents in the hydrogel. Of which, material-substrate interaction was also shown to reduce printed hydrogel width to achieve finer resolution. The finest resolution achieved in this study was 800 μm . A lower resolution is preferred for bioprinting of cell-hydrogel due to a limit of 500 μm for nutrient and waste diffusion. Moreover, it was found that horizontal and vertical grids designed to print at different z layers displayed a collapsed structure. These findings suggest the need to develop a printing strategy that provides better control over print resolution while improving the 3D fidelity of bioprinted construct.

The process of support-assisted bioprinting was developed to enhance print resolution and shape fidelity of bioprinted construct. The cross-sectional profile of support material was studied to control the print resolution of bioink through setting the distance between hydrogel struts while the height of support material is determined for the addition of subsequent z layer. 0°-90° grid structures were printed and have shown the ability of support-assisted bioprinting to produce 3D defined structures. Moreover, 50% reduction in print resolution was achieved using this printing strategy. These advantages of improved resolution and fidelity allows better control over material deposition based on the predefined print path.

7.1.3 Pre-printing technologies for processing data and cells

Novel data processing and file segmentation framework for multi-material deposition in bioprinting was established. This method capitalizes on the tool changing capabilities of bioprinter for multi-material printing and fabricates at a reduced print time. This file preparation approach also provides the basis for generating print path that are more organic and resembles closer to native myocardium. As a proof-of-concept, the print path mimicking fibre orientation of native left ventricle walls was generated and simulated.

Another enabling technology for 3D bioprinting is forming cell aggregates using microfluidic. Due to the complexity of 3D microfluidics design, 3D printing was used to produce these platforms. It was found that intrinsic features such as staircase effects in 3D printing have shown to affect material's properties such as

surface roughness and hydrophilicity of material. This suggests that future studies are needed to develop effective post-processing steps to reduce the intrinsic features. Moreover, sterilization process was established in this study for effective cleansing of the printed parts while assessing the biocompatibility of the 3D printed material.

7.2. Future Work

7.2.1 Characterization of aligned cells

In the current study, bioprinting was investigated and evaluated as a fabrication tool to produce aligned C2C12 constructs. C2C12 cells were directed to align along the longitudinal struts of 0°-90° grid structure. This method of aligning cells has the advantage of creating variation in alignment at each planar layer. However, the current studies focused on the process of directing cell alignment using bioprinting. Future works may include differentiation of aligned C2C12 and assessing the biological and mechanical properties of aligned C2C12 induced via bioprinting. This will verify on the feasibilities of using aligned models from bioprinting for pharmacological evaluation and eventual use in tissue engineering and regenerative medicine.

C2C12 was used in the current study to evaluate on the feasibility in directing cell alignment using bioprinting. As a murine cell line was used in the study, future work may also include exploring different cell types from different sources.

One of the potential cell source for evaluation would be adult stem cells. As adult stem cells require stimulation and differentiation, there is a need to conduct appropriate biological studies to evaluate on the ideal inception point to incorporate these stem cells in the bioprinting process.

7.2.2 Improvement to print resolution model

In the current work, print resolution model was formulated using Power Law models for describing Non-Newtonian Flow. Alginate was used as the analogous material for study. Density of alginate was assumed to be that of water. Future studies may include characterizing different commonly hydrogel materials such as GelMA, collagen, and hyaluronic acid to increase robustness of model. Additionally, the density of hydrogel should be characterized using methods such as dilatometry and pycnometry.

With the proposed bioprinting strategy, 0°-90° grid structures were printed with defined two-layered construct. The support structure effectively provided the z definition to the overall build construct. Consideration for the potential use to recapture the different layers of ventricle wall, there is a need to print more than two layers. Future studies will require investigating the gravitational effect when the number of layers is increased in support-assisted bioprinting.

7.2.3 Algorithms for generating print paths

The G-codes obtained from slicing the ventricle models using various angular planes were performed in a semi-automated manner. The simulated results showed the feasibility of controlling a coordinate-based printing. The predefined print path resembles the orientation of fibres and cells across the transmural wall. Future studies will focus on formulating algorithms that can generate print paths to deposit both build and support materials that conforms to the directional alignment of left ventricular wall.

7.3. List of publications

Published Journal Papers

1. S. Agarwala, J. M. Lee, W. L. Ng, M. Layani, W. Y. Yeong, and S. Magdassi. "A novel 3D bioprinted flexible and biocompatible hydrogel bioelectronic platform." *Biosensors and Bioelectronics*, 102, p 365-371, (2018).
2. W. L. Ng, J. M. Lee, W. Y. Yeong, and M. Win Naing. "Microvalve-based bioprinting—process, bio-inks and applications." *Biomaterials Science*, 5(4), p 632-647, (2017).
3. J. M. Lee, S. L. Sing, E. Y. S. Tan, and W. Y. Yeong, "Bioprinting in cardiovascular tissue engineering: a review." *International Journal of Bioprinting*, 2(2), p 27-36, (2016).
4. J. M. Lee, and W. Y. Yeong. "Design and Printing Strategies in 3D Bioprinting of Cell-Hydrogels: A Review." *Advanced Healthcare Materials*, 5(22), p 2856-2865, (2016).
5. J. M. Lee, M. Zhang, and W. Y. Yeong, "Characterization and Evaluation of Three-Dimensional Printed Microfluidic Chip for Cell Processing", *Microfluidics and Nanofluidics*, 20(1), p 5, (2016). (Highly cited paper as of July/August 2017 in the academic field of Engineering.)
6. S. Wang, J. M. Lee, and W. Y. Yeong, "Smart hydrogels for 3D bioprinting", *International Journal of Bioprinting*, 1(1), (2015).

7. J. M. Lee, and W. Y. Yeong, "A preliminary model of time-pressure dispensing system for bioprinting based on printing and material parameters", *Virtual and Physical Prototyping*, 10(1), p 3-8, (2014).

Submitted Journal Papers

1. J. M. Lee, S. L. Sing, and W. Y. Yeong, "Integrating hydrogel crosslinking mechanisms with CAD/CAM for freeform 3D bioprinting", submitted to *Material Science & Engineering C*.
2. J. M. Lee, and W. Y. Yeong, "Directing cell alignment in support-assisted bioprinting", submitted to *Acta Biomaterialia*.

Book Chapter

1. J. M. Lee, E. Y. S. Tan, Z. Zhu and W. Y. Yeong, "Biomaterials for bioprinting" Book: *3D Bioprinting and Nanotechnology in Tissue Engineering* by Elsevier

Conference Papers and Posters

1. J. M. Lee and W. Y. Yeong, "Bioprinting for cardiovascular tissue engineering," presented at the *2nd International Conference on Pro-AM Progress in Additive Manufacturing*, Singapore, (2016).
2. J. M. Lee and W. Y. Yeong, Poster On "Printing and Material Parameters of Time-Pressure Dispensing System for Bioprinting" presented at the *1st International Bioprinting Congress*, Singapore, (2014). (Best Poster Award)
3. J. M. Lee and W. Y. Yeong, "Understanding Printing and Material Parameters of Time-Pressure Dispensing System for Bioprinting" presented

at the *1st International Conference in Progress in Additive Manufacturing*, Singapore, (2014).

4. J. M. Lee, W. L. Ng, Edgar Tan Yong Sheng, and W. Y. Yeong, Poster on “Bioprinting for Tissue Engineering” presented at the *Bone Tec Congress*, Singapore, (2013).

Patents

1. TD/376/16-3D printed flexible, Layered, Free Standing and Free-Form Bio-Hybrid Electrode (filed, with Singapore Provisional Patent)

References

- [1] E. Braunwald, "Heart failure," *JACC. Heart Failure*, vol. 1, pp. 1-20, Feb 2013.
- [2] A. S. Go, D. Mozaffarian, V. L. Roger, E. J. Benjamin, J. D. Berry, M. J. Blaha, *et al.*, "Heart disease and stroke statistics--2014 update: a report from the American Heart Association," *Circulation*, vol. 129, pp. e28-e292, Jan 21 2014.
- [3] B. Tillman, J. Hardin-Young, W. Shannon, A. J. Russell, and N. L. Parenteau, "Meeting the need for regenerative therapies: translation-focused analysis of U.S. regenerative medicine opportunities in cardiovascular and peripheral vascular medicine using detailed incidence data," *Tissue Engineering Part B: Reviews*, vol. 19, pp. 99-115, Apr 2013.
- [4] A. Lesman, M. Habib, O. Caspi, A. Gepstein, G. Arbel, S. Levenberg, *et al.*, "Transplantation of a Tissue-Engineered Human Vascularized Cardiac Muscle," *Tissue Engineering Part A*, vol. 16, pp. 115-125, Jan 2010.
- [5] S. Levenberg, J. Rouwkema, M. Macdonald, E. S. Garfein, D. S. Kohane, D. C. Darland, *et al.*, "Engineering vascularized skeletal muscle tissue," *Nature Biotechnology*, vol. 23, pp. 879-884, 07//print 2005.
- [6] A. Blazeski, G. M. Kostecki, and L. Tung, "Engineered heart slices for electrophysiological and contractile studies," *Biomaterials*, vol. 55, pp. 119-128, Jul 2015.
- [7] C. Y. Chung, H. Bien, and E. Entcheva, "The role of cardiac tissue alignment in modulating electrical function," *Journal of Cardiovascular Electrophysiology*, vol. 18, pp. 1323-1329, Dec 2007.
- [8] P. L. Kuo, H. Lee, M. A. Bray, N. A. Geisse, Y. T. Huang, W. J. Adams, *et al.*, "Myocyte Shape Regulates Lateral Registry of Sarcomeres and Contractility," *American Journal of Pathology*, vol. 181, pp. 2030-2037, Dec 2012.
- [9] A. J. Pope, G. B. Sands, B. H. Smaill, and I. J. LeGrice, "Three-dimensional transmural organization of perimysial collagen in the heart," *American Journal of Physiology. Heart and Circulatory Physiology*, vol. 295, pp. H1243-H1252, Sep 2008.
- [10] B. Duan, L. A. Hockaday, K. H. Kang, and J. T. Butcher, "3D bioprinting of heterogeneous aortic valve conduits with alginate/gelatin hydrogels," *Journal of Biomedical Materials Research Part A*, vol. 101, pp. 1255-1264, May 2013.

- [11] C. P. Adler, H. Friedburg, G. W. Herget, M. Neuburger, and H. Schwalb, "Variability of cardiomyocyte DNA content, ploidy level and nuclear number in mammalian hearts," *Virchows Archiv*, vol. 429, pp. 159-164, Oct 1996.
- [12] M. A. Laflamme and C. E. Murry, "Heart regeneration," *Nature*, vol. 473, pp. 326-335, 05/19/print 2011.
- [13] D. Sedmera, "Function and form in the developing cardiovascular system," *Cardiovascular Research*, vol. 91, pp. 252-259, Jul 15 2011.
- [14] F. Torrent-Guasp, M. J. Kocica, A. F. Corno, M. Komeda, F. Carreras-Costa, A. Flotats, *et al.*, "Towards new understanding of the heart structure and function," *European Journal of Cardio-Thoracic Surgery*, vol. 27, pp. 191-201, Feb 2005.
- [15] R. H. Anderson, S. Y. Ho, K. Redmann, D. Sanchez-Quintana, and P. P. Lunkenheimer, "The anatomical arrangement of the myocardial cells making up the ventricular mass," *European Journal of Cardio-Thoracic Surgery*, vol. 28, pp. 517-525, Oct 2005.
- [16] S. Y. Ho, "Anatomy and myoarchitecture of the left ventricular wall in normal and in disease," *European Journal of Echocardiography*, vol. 10, pp. iii3-iii7, 2009-12-01 00:00:00 2009.
- [17] P. Hunter, "Cardiovascular Mechanics," in *Encyclopedia of Neuroscience*, M. Binder, N. Hirokawa, and U. Windhorst, Eds., ed: Springer Berlin Heidelberg, 2009, pp. 564-567.
- [18] P. F. Ferreira, P. J. Kilner, L. A. McGill, S. Nielles-Vallespin, A. D. Scott, S. Y. Ho, *et al.*, "In vivo cardiovascular magnetic resonance diffusion tensor imaging shows evidence of abnormal myocardial laminar orientations and mobility in hypertrophic cardiomyopathy," *Journal of Cardiovascular Magnetic Resonance*, vol. 16, p. 87, Nov 12 2014.
- [19] D. Rohmer, A. Sitek, and G. T. Gullberg, "Reconstruction and visualization of fiber and laminar structure in the normal human heart from ex vivo diffusion tensor magnetic resonance imaging (DTMRI) data," *Investigative Radiology*, vol. 42, pp. 777-789, 2007.
- [20] R. Lakshmanan, U. M. Krishnan, and S. Sethuraman, "Living cardiac patch: the elixir for cardiac regeneration," *Expert Opinion on Biological Therapy*, vol. 12, pp. 1623-1640, Dec 2012.
- [21] P. Helm, M. F. Beg, M. I. Miller, and R. L. Winslow, "Measuring and mapping cardiac fiber and laminar architecture using diffusion tensor MR imaging,"

Annals of the New York Academy of Sciences, vol. 1047, pp. 296-307, Jun 2005.

- [22] L. Axel, V. J. Wedeen, and D. B. Ennis, "Probing dynamic myocardial microstructure with cardiac magnetic resonance diffusion tensor imaging," *Journal of Cardiovascular Magnetic Resonance*, vol. 16, p. 89, Nov 12 2014.
- [23] M. G. S. J. Sutton and N. Sharpe, "Left Ventricular Remodeling After Myocardial Infarction: Pathophysiology and Therapy," *Circulation*, vol. 101, pp. 2981-2988, June 27, 2000 2000.
- [24] M. C. Alraies and P. Eckman, "Adult heart transplant: indications and outcomes," *Journal of Thoracic Disease*, vol. 6, pp. 1120-1128, 2014.
- [25] A. Shafiee and A. Atala, "Tissue Engineering: Toward a New Era of Medicine," *Annual Review of Medicine*, vol. 68, pp. 29-40, 2017.
- [26] C. Mandrycky, Z. Wang, K. Kim, and D.-H. Kim, "3D bioprinting for engineering complex tissues," *Biotechnology Advances*, vol. 34, pp. 422-434, 2016/07/01/ 2016.
- [27] R. R. Makkar, R. R. Smith, K. Cheng, K. Malliaras, L. E. J. Thomson, D. Berman, *et al.*, "Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial," *The Lancet*, vol. 379, pp. 895-904, 2012.
- [28] R. Bolli, A. R. Chugh, D. D'Amario, J. H. Loughran, M. F. Stoddard, S. Ikram, *et al.*, "Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial," *The Lancet*, vol. 378, pp. 1847-1857, Nov 26 2011.
- [29] A. Skardal, J. Zhang, L. McCoard, X. Xu, S. Oottamasathien, and G. D. Prestwich, "Photocrosslinkable Hyaluronan-Gelatin Hydrogels for Two-Step Bioprinting," *Tissue Engineering Part A*, vol. 16, pp. 2675-2685, 2010.
- [30] K. Malliaras, R. R. Makkar, R. R. Smith, K. Cheng, E. Wu, R. O. Bonow, *et al.*, "Intracoronary cardiosphere-derived cells after myocardial infarction: evidence of therapeutic regeneration in the final 1-year results of the CADUCEUS trial (CARDiosphere-Derived aUtologous stem CELls to reverse ventricUlar dySfunction)," *Journal of the American College of Cardiology*, vol. 63, pp. 110-122, Jan 21 2014.
- [31] K. Cheng, A. Blusztajn, D. Shen, T. S. Li, B. Sun, G. Galang, *et al.*, "Functional performance of human cardiosphere-derived cells delivered in an in situ polymerizable hyaluronan-gelatin hydrogel," *Biomaterials*, vol. 33, pp. 5317-24, Jul 2012.

- [32] B. A. Aguado, W. Mulyasasmita, J. Su, K. J. Lampe, and S. C. Heilshorn, "Improving viability of stem cells during syringe needle flow through the design of hydrogel cell carriers," *Tissue Engineering Part A*, vol. 18, pp. 806-815, Apr 2012.
- [33] D. Zhang, I. Y. Shadrin, J. Lam, H.-Q. Xian, H. R. Snodgrass, and N. Bursac, "Tissue-engineered cardiac patch for advanced functional maturation of human ESC-derived cardiomyocytes," *Biomaterials*, vol. 34, pp. 5813-5820, 7// 2013.
- [34] F. Weinberger, I. Mannhardt, and T. Eschenhagen, "Engineering Cardiac Muscle Tissue A Maturing Field of Research," *Circulation Research*, vol. 120, pp. 1487-1500, Apr 2017.
- [35] G. V. Novakovic, T. Eschenhagen, and C. Mummery, "Myocardial Tissue Engineering: In Vitro Models," *Cold Spring Harbor Perspectives in Medicine*, vol. 4, p. a014076, Mar 2014.
- [36] R. P. Davis, S. Casini, C. W. van den Berg, M. Hoekstra, C. A. Remme, C. Dambrot, *et al.*, "Cardiomyocytes derived from pluripotent stem cells recapitulate electrophysiological characteristics of an overlap syndrome of cardiac sodium channel disease," *Circulation*, vol. 125, pp. 3079-3091, Jun 26 2012.
- [37] H. Jawad, N. N. Ali, A. R. Lyon, Q. Z. Chen, S. E. Harding, and A. R. Boccaccini, "Myocardial tissue engineering: a review," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 1, pp. 327-342, Sep-Oct 2007.
- [38] R. K. Iyer, L. L. Chiu, L. A. Reis, and M. Radisic, "Engineered cardiac tissues," *Current Opinion in Biotechnology*, vol. 22, pp. 706-714, Oct 2011.
- [39] G. Vunjak-Novakovic, K. O. Lui, N. Tandon, and K. R. Chien, "Bioengineering heart muscle: a paradigm for regenerative medicine," *Annual Review of Biomedical engineering*, vol. 13, pp. 245-267, Aug 15 2011.
- [40] A. R. Pinto, A. Ilinykh, M. J. Ivey, J. T. Kuwabara, M. L. D'Antoni, R. Debuque, *et al.*, "Revisiting Cardiac Cellular Composition," *Circulation Research*, vol. 118, pp. 400-409, 12/03 2016.
- [41] P. Kohl, P. Camelliti, F. L. Burton, and G. L. Smith, "Electrical coupling of fibroblasts and myocytes: relevance for cardiac propagation," *Journal of Electrocardiology*, vol. 38, pp. 45-50, 10// 2005.
- [42] P. Camelliti, C. R. Green, I. LeGrice, and P. Kohl, "Fibroblast Network in Rabbit Sinoatrial Node: Structural and Functional Identification of Homogeneous and Heterogeneous Cell Coupling," *Circulation Research*, vol. 94, pp. 828-835, April 2, 2004 2004.

- [43] E. Hirsch, R. Nagai, and T. Thum, "Heterocellular signalling and crosstalk in the heart in ischaemia and heart failure," *Cardiovascular Research*, vol. 102, pp. 191-193, 2014-05-01 00:00:00 2014.
- [44] H. Sekine, T. Shimizu, K. Hobo, S. Sekiya, J. Yang, M. Yamato, *et al.*, "Endothelial Cell Coculture Within Tissue-Engineered Cardiomyocyte Sheets Enhances Neovascularization and Improves Cardiac Function of Ischemic Hearts," *Circulation*, vol. 118, pp. S145-S152, September 30, 2008 2008.
- [45] R. K. Iyer, L. L. Y. Chiu, and M. Radisic, "Microfabricated poly(ethylene glycol) templates enable rapid screening of triculture conditions for cardiac tissue engineering," *Journal of Biomedical Materials Research Part A*, vol. 89, pp. 616-631, 2009.
- [46] B. Wang, G. Wang, F. To, J. R. Butler, A. Claude, R. M. McLaughlin, *et al.*, "Myocardial Scaffold-based Cardiac Tissue Engineering: Application of Coordinated Mechanical and Electrical Stimulations," *Langmuir : the ACS journal of surfaces and colloids*, vol. 29, p. 10.1021/la401702w, 08/20 2013.
- [47] C.-W. Hsiao, M.-Y. Bai, Y. Chang, M.-F. Chung, T.-Y. Lee, C.-T. Wu, *et al.*, "Electrical coupling of isolated cardiomyocyte clusters grown on aligned conductive nanofibrous meshes for their synchronized beating," *Biomaterials*, vol. 34, pp. 1063-1072, 1// 2013.
- [48] G. Vunjak-Novakovic, N. Tandon, A. Godier, R. Maidhof, A. Marsano, T. P. Martens, *et al.*, "Challenges in Cardiac Tissue Engineering," *Tissue Engineering Part B: Reviews*, vol. 16, pp. 169-187, Apr 2010.
- [49] K. Sakaguchi, T. Shimizu, S. Horaguchi, H. Sekine, M. Yamato, M. Umezu, *et al.*, "In Vitro Engineering of Vascularized Tissue Surrogates," *Scientific Reports*, vol. 3, p. 1316, 02/19/online 2013.
- [50] H. Hata, A. Bar, S. Dorfman, Z. Vukadinovic, Y. Sawa, A. Haverich, *et al.*, "Engineering a novel three-dimensional contractile myocardial patch with cell sheets and decellularised matrix," *European Journal of Cardio-thoracic Surgery*, vol. 38, pp. 450-455, Oct 2010.
- [51] S. Nakatani, "Left Ventricular Rotation and Twist: Why Should We Learn?," *Journal of Cardiovascular Ultrasound*, vol. 19, pp. 1-6, 03/31 2011.
- [52] C. Mekkaoui, S. Huang, H. Chen, G. Dai, T. Reese, W. Kostis, *et al.*, "Fiber architecture in remodeled myocardium revealed with a quantitative diffusion CMR tractography framework and histological validation," *Journal of Cardiovascular Magnetic Resonance*, vol. 14, p. 70, 2012.

- [53] M. C. Ribeiro, L. G. Tertoolen, J. A. Guadix, M. Bellin, G. Kosmidis, C. D'Aniello, *et al.*, "Functional maturation of human pluripotent stem cell derived cardiomyocytes in vitro - Correlation between contraction force and electrophysiology," *Biomaterials*, vol. 51, pp. 138-150, May 2015.
- [54] K. Nagase, M. Yamato, H. Kanazawa, and T. Okano, "Poly(N-isopropylacrylamide)-based thermoresponsive surfaces provide new types of biomedical applications," *Biomaterials*, vol. 153, pp. 27-48, 2018/01/01/ 2018.
- [55] T. Shimizu, M. Yamato, A. Kikuchi, and T. Okano, "Cell sheet engineering for myocardial tissue reconstruction," *Biomaterials*, vol. 24, pp. 2309-2316, 6// 2003.
- [56] K. Miki, H. Uenaka, A. Saito, S. Miyagawa, T. Sakaguchi, T. Higuchi, *et al.*, "Bioengineered myocardium derived from induced pluripotent stem cells improves cardiac function and attenuates cardiac remodeling following chronic myocardial infarction in rats," *Stem Cells Translational Medicine*, vol. 1, pp. 430-437, May 2012.
- [57] C. E. LeBlon, R. Pai, C. R. Fodor, A. S. Golding, J. P. Coulter, and S. S. Jedlicka, "In vitro comparative biodegradation analysis of salt-leached porous polymer scaffolds," *Journal of Applied Polymer Science*, vol. 128, pp. 2701-2712, Jun 2013.
- [58] D. Sin, X. G. Miao, G. Liu, F. Wei, G. Chadwick, C. Yan, *et al.*, "Polyurethane (PU) scaffolds prepared by solvent casting/particulate leaching (SCPL) combined with centrifugation," *Materials Science & Engineering C-Materials for Biological Applications*, vol. 30, pp. 78-85, Jan 2010.
- [59] A. P. Pego, B. Siebum, M. J. A. Van Luyn, X. Van Seijen, A. A. Poot, D. W. Grijpma, *et al.*, "Preparation of degradable porous structures based on 1,3-trimethylene carbonate and D,L-lactide (co)polymers for heart tissue engineering," *Tissue Engineering*, vol. 9, pp. 981-994, Oct 2003.
- [60] R. A. Neal, A. Jean, H. Park, P. B. Wu, J. Hsiao, G. C. Engelmayr, Jr., *et al.*, "Three-dimensional elastomeric scaffolds designed with cardiac-mimetic structural and mechanical features," *Tissue Engineering Part A*, vol. 19, pp. 793-807, Mar 2013.
- [61] M. Kharaziha, M. Nikkhah, S. R. Shin, N. Annabi, N. Masoumi, A. K. Gaharwar, *et al.*, "PGS:Gelatin nanofibrous scaffolds with tunable mechanical and structural properties for engineering cardiac tissues," *Biomaterials*, vol. 34, pp. 6355-6366, Sep 2013.

- [62] Y. Orlova, N. Magome, L. Liu, Y. Chen, and K. Agladze, "Electrospun nanofibers as a tool for architecture control in engineered cardiac tissue," *Biomaterials*, vol. 32, pp. 5615-5624, Aug 2011.
- [63] T. Xu, W. Zhao, J. M. Zhu, M. Z. Albanna, J. J. Yoo, and A. Atala, "Complex heterogeneous tissue constructs containing multiple cell types prepared by inkjet printing technology," *Biomaterials*, vol. 34, pp. 130-139, Jan 2013.
- [64] M. P. Prabhakaran, A. S. Nair, D. Kai, and S. Ramakrishna, "Electrospun composite scaffolds containing poly(octanediol-co-citrate) for cardiac tissue engineering," *Biopolymers*, vol. 97, pp. 529-538, Jul 2012.
- [65] D. Kai, M. P. Prabhakaran, G. Jin, and S. Ramakrishna, "Guided orientation of cardiomyocytes on electrospun aligned nanofibers for cardiac tissue engineering," *Journal of Biomedical Materials Research Part B, Applied Biomaterials*, vol. 98, pp. 379-386, Aug 2011.
- [66] M. Shevach, B. M. Maoz, R. Feiner, A. Shapira, and T. Dvir, "Nanoengineering gold particle composite fibers for cardiac tissue engineering," *Journal of Materials Chemistry B*, vol. 1, pp. 5210-5217, 2013.
- [67] S. Fleischer, R. Feiner, A. Shapira, J. Ji, X. Sui, H. D. Wagner, *et al.*, "Spring-like fibers for cardiac tissue engineering," *Biomaterials*, vol. 34, pp. 8599-8606, Nov 2013.
- [68] W. Y. Yeong, N. Sudarmadji, H. Y. Yu, C. K. Chua, K. F. Leong, S. S. Venkatraman, *et al.*, "Porous polycaprolactone scaffold for cardiac tissue engineering fabricated by selective laser sintering," *Acta Biomaterialia*, vol. 6, pp. 2028-2034, Jun 2010.
- [69] G. C. Engelmayr, Jr., M. Cheng, C. J. Bettinger, J. T. Borenstein, R. Langer, and L. E. Freed, "Accordion-like honeycombs for tissue engineering of cardiac anisotropy," *Nature Materials*, vol. 7, pp. 1003-1010, Dec 2008.
- [70] S. Pok, J. D. Myers, S. V. Madhally, and J. G. Jacot, "A multilayered scaffold of a chitosan and gelatin hydrogel supported by a PCL core for cardiac tissue engineering," *Acta Biomaterialia*, vol. 9, pp. 5630-5642, Mar 2013.
- [71] D. Kai, M. P. Prabhakaran, G. Jin, and S. Ramakrishna, "Guided orientation of cardiomyocytes on electrospun aligned nanofibers for cardiac tissue engineering," *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, vol. 98B, pp. 379-386, 2011.
- [72] A. G. Guex, A. Frobert, J. Valentin, G. Fortunato, D. Hegemann, S. Cook, *et al.*, "Plasma-functionalized electrospun matrix for biograft development and cardiac function stabilization," *Acta Biomaterialia*, vol. 10, pp. 2996-3006, 2014/07/01/ 2014.

- [73] S. Wu, B. Duan, X. Qin, and J. T. Butcher, "Nanofiber-structured hydrogel yarns with pH-response capacity and cardiomyocyte-drivability for bio-microactuator application," *Acta Biomaterialia*, vol. 60, pp. 144-153, 2017/09/15/ 2017.
- [74] M. Castilho, D. Feyen, M. Flandes - Iparraguirre, G. Hochleitner, J. Groll, P. A. F. Doevendans, *et al.*, "Melt Electrospinning Writing of Poly - Hydroxymethylglycolide - co - ϵ - Caprolactone - Based Scaffolds for Cardiac Tissue Engineering," *Advanced Healthcare Materials*, vol. 6, p. 1700311, 2017.
- [75] W. H. Zimmermann, C. Fink, D. Kralisch, U. Remmers, J. Weil, and T. Eschenhagen, "Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes," *Biotechnology and Bioengineering*, vol. 68, pp. 106-114, Apr 5 2000.
- [76] W. H. Zimmermann, I. Melnychenko, G. Wasmeier, M. Didie, H. Naito, U. Nixdorff, *et al.*, "Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts," *Nature Medicine*, vol. 12, pp. 452-8, Apr 2006.
- [77] I. Y. Shadrin, B. W. Allen, Y. Qian, C. P. Jackman, A. L. Carlson, M. E. Juhas, *et al.*, "Cardiopatch platform enables maturation and scale-up of human pluripotent stem cell-derived engineered heart tissues," *Nature Communications*, vol. 8, p. 1825, 2017/11/28 2017.
- [78] C. Y. Li, D. K. Wood, C. M. Hsu, and S. N. Bhatia, "DNA-templated assembly of droplet-derived PEG microtissues," *Lab on a Chip*, vol. 11, pp. 2967-2975, Sep 7 2011.
- [79] P. M. Dohmen, M. Scheckel, M. Stein-Konertz, W. Erdbruegger, K. Affeld, and W. Konertz, "In vitro hydrodynamics of a decellularized pulmonary porcine valve, compared with a glutaraldehyde and polyurethane heart valve," *International Journal of Artificial Organs*, vol. 25, pp. 1089-1094, Nov 2002.
- [80] F. Juthier, A. Vincentelli, J. Gaudric, D. Corseaux, O. Fouquet, C. Calet, *et al.*, "Decellularized heart valve as a scaffold for in vivo recellularization: Deleterious effects of granulocyte colony-stimulating factor," *Journal of Thoracic and Cardiovascular Surgery*, vol. 131, pp. 843-852, Apr 2006.
- [81] H. C. Ott, T. S. Matthiesen, S.-K. Goh, L. D. Black, S. M. Kren, T. I. Netoff, *et al.*, "Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart," *Nature Medicine*, vol. 14, pp. 213-221, Feb 2008.

- [82] T. Shimizu, "Cell Sheet-Based Tissue Engineering for Fabricating 3-Dimensional Heart Tissues," *Circulation Journal*, vol. 78, pp. 2594-2603, 2014.
- [83] H. Yasui, J.-K. Lee, A. Yoshida, T. Yokoyama, H. Nakanishi, K. Miwa, *et al.*, "Excitation propagation in three-dimensional engineered hearts using decellularized extracellular matrix," *Biomaterials*, vol. 35, pp. 7839-7850, 9// 2014.
- [84] T. Dvir, B. P. Timko, M. D. Brigham, S. R. Naik, S. S. Karajanagi, O. Levy, *et al.*, "Nanowired three-dimensional cardiac patches," *Nature Nanotechnology*, vol. 6, pp. 720-725, Nov 2011.
- [85] V. Mironov, R. P. Visconti, V. Kasyanov, G. Forgacs, C. J. Drake, and R. R. Markwald, "Organ printing: Tissue spheroids as building blocks," *Biomaterials*, vol. 30, pp. 2164-2174, 2009.
- [86] R. A. Rezende, F. D. A. S. Pereira, V. Kasyanov, D. T. Kemmoku, I. Maia, J. V. L. da Silva, *et al.*, "Scalable Biofabrication of Tissue Spheroids for Organ Printing," *Procedia CIRP*, vol. 5, pp. 276-281, 2013.
- [87] J. M. Kelm, N. E. Timmins, C. J. Brown, M. Fussenegger, and L. K. Nielsen, "Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types," *Biotechnology and Bioengineering*, vol. 83, pp. 173-180, 2003.
- [88] R. Z. Lin and H. Y. Chang, "Recent advances in three-dimensional multicellular spheroid culture for biomedical research," *Biotechnology Journal*, vol. 3, pp. 1172-1184, Oct 2008.
- [89] A. Nagelkerke, J. Bussink, F. Sweep, and P. N. Span, "Generation of multicellular tumor spheroids of breast cancer cells: How to go three-dimensional," *Analytical Biochemistry*, vol. 437, pp. 17-19, Jun 2013.
- [90] W. G. Lee, D. Ortmann, M. J. Hancock, H. Bae, and A. Khademhosseini, "A hollow sphere soft lithography approach for long-term hanging drop methods," *Tissue Engineering Part C: Methods*, vol. 16, pp. 249-259, 2010.
- [91] A. Y. Hsiao, Y. C. Tung, C. H. Kuo, B. Mosadegh, R. Bedenis, K. J. Pienta, *et al.*, "Micro-ring structures stabilize microdroplets to enable long term spheroid culture in 384 hanging drop array plates," *Biomedical Microdevices*, vol. 14, pp. 313-323, Apr 2012.
- [92] J. M. Karp, J. Yen, G. Eng, J. Fukuda, J. Blumling, S. Kahp-Yang, *et al.*, "Controlling size, shape and homogeneity of embryoid bodies using poly(ethylene glycol) microwells," *Lab on a Chip*, vol. 4, pp. 786-794, 2007.

- [93] Y.-s. Torisawa, A. Takagi, Y. Nashimoto, T. Yasukawa, H. Shiku, and T. Matsue, "A multicellular spheroid array to and viability realize spheroid formation, culture, assay on a chip," *Biomaterials*, vol. 28, pp. 559-566, Jan 2007.
- [94] H. F. Chan, Y. Zhang, Y.-P. Ho, Y.-L. Chiu, Y. Jung, and K. W. Leong, "Rapid formation of multicellular spheroids in double-emulsion droplets with controllable microenvironment," *Scientific Reports*, vol. 3, p. 3462, 2013.
- [95] K. Watanabe, T. Fujiyama, R. Mitsutake, M. Watanabe, Y. Tazaki, T. Miyazaki, *et al.*, "Fabrication of Growth Factor Array Using an Inkjet Printer," in *Cell and Organ Printing*, S. B. Ringeisen B., Wu P. , Ed., ed: Springer, Dordrecht, 2010, pp. 203-222.
- [96] K. Alessandri, B. R. Sarangi, V. V. Gurchenkov, B. Sinha, T. R. Kiessling, L. Fetler, *et al.*, "Cellular capsules as a tool for multicellular spheroid production and for investigating the mechanics of tumor progression in vitro," *Proceedings of the National Academy of Sciences*, vol. 110, pp. 14843-14848, Sep 10 2013.
- [97] K. Lee, C. Kim, J. Y. Yang, H. Lee, B. Ahn, L. Xu, *et al.*, "Gravity-oriented microfluidic device for uniform and massive cell spheroid formation," *Biomicrofluidics*, vol. 6, p. 014114, Mar 2012.
- [98] B. Patra, Y.-H. Chen, C.-C. Peng, S.-C. Lin, C.-H. Lee, and Y.-C. Tung, "A microfluidic device for uniform-sized cell spheroids formation, culture, harvesting and flow cytometry analysis," *Biomicrofluidics*, vol. 7, p. 054114, Sep 2013.
- [99] K. Ziolkowska, R. Kwapiszewski, A. Stelmachowska, M. Chudy, A. Dybko, and Z. Brzozka, "Development of a three-dimensional microfluidic system for long-term tumor spheroid culture," *Sensors and Actuators B: Chemical*, vol. 173, pp. 908-913, Oct 2012.
- [100] C. Kim, J. H. Bang, Y. E. Kim, S. H. Lee, and J. Y. Kang, "On-chip anticancer drug test of regular tumor spheroids formed in microwells by a distributive microchannel network," *Lab on a Chip*, vol. 12, pp. 4135-4142, 2012 2012.
- [101] J. Warrick, B. Casavant, M. Frisk, and D. Beebe, "A microfluidic cell concentrator," *Analytical Chemistry*, vol. 82, pp. 8320-8326, 2010.
- [102] T. Okuyama, H. Yamazoe, N. Mochizuki, A. Khademhosseini, H. Suzuki, and J. Fukuda, "Preparation of arrays of cell spheroids and spheroid-monolayer cocultures within a microfluidic device," *Journal of Bioscience and Bioengineering*, vol. 110, pp. 572-576, Nov 2010.

- [103] L. Y. Wu, D. Di Carlo, and L. P. Lee, "Microfluidic self-assembly of tumor spheroids for anticancer drug discovery," *Biomedical Microdevices*, vol. 10, pp. 197-202, Apr 2008.
- [104] H. Tekin, M. Anaya, M. D. Brigham, C. Nauman, R. Langer, and A. Khademhosseini, "Stimuli-responsive microwells for formation and retrieval of cell aggregates," *Lab on a Chip*, vol. 10, pp. 2411-2418, 2010 2010.
- [105] H. Tekin, G. Ozaydin-Ince, T. Tsinman, K. K. Gleason, R. Langer, A. Khademhosseini, *et al.*, "Responsive microgrooves for the formation of harvestable tissue constructs," *Langmuir*, vol. 27, pp. 5671-5679, 2011.
- [106] H. Ota, T. Kodama, and N. Miki, "Rapid formation of size-controlled three dimensional hetero-cell aggregates using micro-rotation flow for spheroid study," *Biomicrofluidics*, vol. 5, p. 034105, Sep 2011.
- [107] H. Ota, T. Kodama, and N. Miki, "Microfluidic experimental array using micro-rotation flow for producing size-controlled three-dimensional spheroids," in *International Symposium on Micro-NanoMechatronics and Human Science (MHS)*, Nagoya, Japan, 2010, pp. 362-366.
- [108] A. Y. Hsiao, Y.-s. Torisawa, Y.-C. Tung, S. Sud, R. S. Taichman, K. J. Pienta, *et al.*, "Microfluidic system for formation of PC-3 prostate cancer co-culture spheroids," *Biomaterials*, vol. 30, pp. 3020-3027, Jun 2009.
- [109] H. Takai, M. Kojima, K. Ohara, M. Horade, T. Tanikawa, Y. Mae, *et al.*, "Microfluidic Device for Automated Generation of Toroidal-like Spheroids," in *10th International Conference on Ubiquitous Robots and Ambient Intelligence (URAI)*, Jeju, South Korea, 2013, pp. 140-143.
- [110] C.-T. Kuo, C.-L. Chiang, R. Y.-J. Huang, H. Lee, and A. M. Wo, "Configurable 2D and 3D spheroid tissue cultures on bioengineered surfaces with acquisition of epithelial-mesenchymal transition characteristics," *NPG Asia Materials*, vol. 4, p. e27, Sep 2012.
- [111] Y.-s. Torisawa, B. Mosadegh, G. D. Luker, M. Morell, K. S. O'Shea, and S. Takayama, "Microfluidic hydrodynamic cellular patterning for systematic formation of co-culture spheroids," *Integrative Biology*, vol. 1, pp. 649-654, 2009 2009.
- [112] Y. Akiyama and K. Morishima, "Label-free cell aggregate formation based on the magneto-Archimedes effect," *Applied Physics Letters*, vol. 98, p. 163702, 2011.
- [113] A. Bonyar, H. Santha, M. Varga, B. Ring, A. Vitez, and G. Harsanyi, "Characterization of rapid PDMS casting technique utilizing molding forms

fabricated by 3D rapid prototyping technology (RPT)," *International Journal of Material Forming*, vol. 7, pp. 189-196, Jun 2014.

- [114] H. F. Chan, Y. Zhang, Y.-P. Ho, Y.-L. Chiu, Y. Jung, and K. W. Leong, "Rapid formation of multicellular spheroids in double-emulsion droplets with controllable microenvironment," *Scientific Reports*, vol. 3, 2013.
- [115] K. H. Lee, D. Y. No, S. H. Kim, J. H. Ryoo, S. F. Wong, and S. H. Lee, "Diffusion-mediated in situ alginate encapsulation of cell spheroids using microscale concave well and nanoporous membrane," *Lab on a Chip*, vol. 11, pp. 1168-1173, 2011.
- [116] M. Papadaki, N. Bursac, R. Langer, J. Merok, G. Vunjak-Novakovic, and L. E. Freed, "Tissue engineering of functional cardiac muscle: molecular, structural, and electrophysiological studies," *American Journal of Physiology. Heart and Circulatory Physiology*, vol. 280, pp. H168-78, Jan 2001.
- [117] P. M. Wigmore and G. F. Dunlison, "The generation of fiber diversity during myogenesis," *International Journal of Developmental Biology*, vol. 42, pp. 117-125, Mar 1998.
- [118] P. Friedl, E. Sahai, S. Weiss, and K. M. Yamada, "New dimensions in cell migration," *Nature Reviews Molecular Cell Biology*, vol. 13, pp. 743-747, Nov 2012.
- [119] L. Wang, Y. H. Li, B. Chen, S. B. Liu, M. X. Li, L. Zheng, *et al.*, "Patterning Cellular Alignment through Stretching Hydrogels with Programmable Strain Gradients," *ACS Applied Materials & Interfaces*, vol. 7, pp. 15088-15097, Jul 15 2015.
- [120] M. Flaibani, L. Boldrin, E. Cimetta, M. Piccoli, P. De Coppi, and N. Elvassore, "Muscle Differentiation and Myotubes Alignment Is Influenced by Micropatterned Surfaces and Exogenous Electrical Stimulation," *Tissue Engineering Part A*, vol. 15, pp. 2447-2457, Sep 2009.
- [121] Z. Zhao, L. Qin, B. Reid, J. Pu, T. Hara, and M. Zhao, "Directing migration of endothelial progenitor cells with applied DC electric fields," *Stem Cell Research*, vol. 8, pp. 38-48, 2012/01/01/ 2012.
- [122] M. Radisic, H. Park, H. Shing, T. Consi, F. J. Schoen, R. Langer, *et al.*, "Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds," *Proceedings of the National Academy of Sciences*, vol. 101, pp. 18129-18134, Dec 28 2004.
- [123] A. Lluica-Valdeperas, B. Sanchez, C. Soler-Botija, C. Galvez-Monton, C. Prat-Vidal, S. Roura, *et al.*, "Electrical stimulation of cardiac adipose tissue-

derived progenitor cells modulates cell phenotype and genetic machinery," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 9, pp. E76-E83, Nov 2015.

- [124] J. Foolen, V. S. Deshpande, F. M. W. Kanters, and F. P. T. Baaijens, "The influence of matrix integrity on stress-fiber remodeling in 3D," *Biomaterials*, vol. 33, pp. 7508-7518, Oct 2012.
- [125] S. Chiron, C. Tomczak, A. Duperray, J. Laine, G. Bonne, A. Eder, *et al.*, "Complex Interactions between Human Myoblasts and the Surrounding 3D Fibrin-Based Matrix," *Plos One*, vol. 7, p. e36173, Apr 2012.
- [126] W. Bian, M. Juhas, T. W. Pfeiler, and N. Bursac, "Local tissue geometry determines contractile force generation of engineered muscle networks," *Tissue Engineering Part A*, vol. 18, pp. 957-967, May 2012.
- [127] B. Weining, P. J. Christopher, and B. Nenad, "Controlling the structural and functional anisotropy of engineered cardiac tissues," *Biofabrication*, vol. 6, p. 024109, 2014.
- [128] W. Bian, B. Liao, N. Badie, and N. Bursac, "Mesoscopic hydrogel molding to control the 3D geometry of bioartificial muscle tissues," *Nature Protocols*, vol. 4, pp. 1522-1534, 09//print 2009.
- [129] H. Aubin, J. W. Nichol, C. B. Hutson, H. Bae, A. L. Sieminski, D. M. Cropek, *et al.*, "Directed 3D cell alignment and elongation in microengineered hydrogels," *Biomaterials*, vol. 31, pp. 6941-6951, Sep 2010.
- [130] S. M. Gopalan, C. Flaim, S. N. Bhatia, M. Hoshijima, R. Knoell, K. R. Chien, *et al.*, "Anisotropic stretch-induced hypertrophy in neonatal ventricular myocytes micropatterned on deformable elastomers," *Biotechnology and Bioengineering*, vol. 81, pp. 578-587, 2003.
- [131] C. C. Winter, K. S. Katiyar, N. S. Hernandez, Y. J. Song, L. A. Struzyna, J. P. Harris, *et al.*, "Transplantable living scaffolds comprised of micro-tissue engineered aligned astrocyte networks to facilitate central nervous system regeneration," *Acta Biomaterialia*, vol. 38, pp. 44-58, Jul 01 2016.
- [132] B. Sun, K. Xie, T.-H. Chen, and R. H. W. Lam, "Preferred cell alignment along concave microgrooves," *RSC Advances*, vol. 7, pp. 6788-6794, 2017.
- [133] H. Takahashi and T. Okano, "Cell Sheet-Based Tissue Engineering for Organizing Anisotropic Tissue Constructs Produced Using Microfabricated Thermoresponsive Substrates," *Advanced Healthcare Materials*, vol. 4, pp. 2388-2407, 2015.

- [134] B. Zhang, Y. Xiao, A. Hsieh, N. Thavandiran, and M. Radisic, "Micro-and nanotechnology in cardiovascular tissue engineering," *Nanotechnology*, vol. 22, p. 494003, 2011.
- [135] L. Wang, Y. B. Wu, B. L. Guo, and P. X. Ma, "Nanofiber Yarn/Hydrogel Core-Shell Scaffolds Mimicking Native Skeletal Muscle Tissue for Guiding 3D Myoblast Alignment, Elongation, and Differentiation," *ACS Nano*, vol. 9, pp. 9167-9179, Sep 2015.
- [136] S. H. Kim, S.-K. Im, S.-J. Oh, S. Jeong, E.-S. Yoon, C. J. Lee, *et al.*, "Anisotropically organized three-dimensional culture platform for reconstruction of a hippocampal neural network," *Nature Communications*, vol. 8, p. 14346, 02/01/online 2017.
- [137] A. Atmanli and I. Domian, "Generation of Aligned 2-Dimensional Myocardial Tissue From Committed Ventricular Progenitors," *Journal of Visualized Experiments : JoVE*, pp. e50288-e50288, 03/19 2013.
- [138] Y. H. Li, G. Y. Huang, M. X. Li, L. Wang, E. L. Elson, T. J. Lu, *et al.*, "An approach to quantifying 3D responses of cells to extreme strain," *Scientific Reports*, vol. 6, p. 19550, Feb 18 2016.
- [139] Y. Li, G. Huang, X. Zhang, L. Wang, Y. Du, T. J. Lu, *et al.*, "Engineering cell alignment in vitro," *Biotechnology Advances*, vol. 32, pp. 347-365, Mar-Apr 2014.
- [140] F. Guillemot, V. Mironov, and M. Nakamura, "Bioprinting is coming of age: Report from the International Conference on Bioprinting and Biofabrication in Bordeaux (3B'09)," *Biofabrication*, vol. 2, p. 010201, Mar 2010.
- [141] J. Korpela, A. Kokkari, H. Korhonen, M. Malin, T. Narhi, and J. Seppala, "Biodegradable and bioactive porous scaffold structures prepared using fused deposition modeling," *Journal of Biomedical Materials Research Part B-Applied Biomaterials*, vol. 101B, pp. 610-619, May 2013.
- [142] F. P. W. Melchels, J. Feijen, and D. W. Grijpma, "A poly(D,L-lactide) resin for the preparation of tissue engineering scaffolds by stereolithography," *Biomaterials*, vol. 30, pp. 3801-3809, Aug 2009.
- [143] D. J. Odde and M. J. Renn, "Laser-guided direct writing for applications in biotechnology," *Trends in Biotechnology*, vol. 17, pp. 385-389, 10/1/ 1999.
- [144] A. Abeyewickreme, A. Kwok, J. R. McEwan, and S. N. Jayasinghe, "Bio-electrospraying embryonic stem cells: interrogating cellular viability and pluripotency," *Integrative Biology*, vol. 1, pp. 260-266, Mar 2009.

- [145] Y. Fang, J. P. Frampton, S. Raghavan, R. Sabahi-Kaviani, G. Luker, C. X. Deng, *et al.*, "Rapid generation of multiplexed cell cocultures using acoustic droplet ejection followed by aqueous two-phase exclusion patterning," *Tissue Engineering Part C: Methods*, vol. 18, pp. 647-657, Sep 2012.
- [146] F. Guillemot, B. Guillotin, A. Fontaine, M. Ali, S. Catros, V. Keriquel, *et al.*, "Laser-assisted bioprinting to deal with tissue complexity in regenerative medicine," *Mrs Bulletin*, vol. 36, pp. 1015-1019, Dec 2011.
- [147] B. Guillotin, A. Souquet, S. Catros, M. Duocastella, B. Pippenger, S. Bellance, *et al.*, "Laser assisted bioprinting of engineered tissue with high cell density and microscale organization," *Biomaterials*, vol. 31, pp. 7250-7256, Oct 2010.
- [148] R. Gaebel, N. Ma, J. Liu, J. Guan, L. Koch, C. Klopsch, *et al.*, "Patterning human stem cells and endothelial cells with laser printing for cardiac regeneration," *Biomaterials*, vol. 32, pp. 9218-9230, Dec 2011.
- [149] S. Ahn, H. Lee, and G. Kim, "Functional cell-laden alginate scaffolds consisting of core/shell struts for tissue regeneration," *Carbohydrate Polymers*, vol. 98, pp. 936-942, Oct 15 2013.
- [150] T. Billiet, E. Gevaert, T. De Schryver, M. Cornelissen, and P. Dubruel, "The 3D printing of gelatin methacrylamide cell-laden tissue-engineered constructs with high cell viability," *Biomaterials*, vol. 35, pp. 49-62, Jan 2014.
- [151] N. E. Fedorovich, H. M. Wijnberg, W. J. Dhert, and J. Alblas, "Distinct tissue formation by heterogeneous printing of osteo- and endothelial progenitor cells," *Tissue Engineering Part A*, vol. 17, pp. 2113-21, Aug 2011.
- [152] Y. Huang, K. He, and X. Wang, "Rapid prototyping of a hybrid hierarchical polyurethane-cell/hydrogel construct for regenerative medicine," *Materials Science & Engineering C-Materials for Biological Applications*, vol. 33, pp. 3220-3229, Aug 1 2013.
- [153] H. Lee, S. Ahn, L. J. Bonassar, W. Chun, and G. Kim, "Cell-laden poly(ϵ -caprolactone)/alginate hybrid scaffolds fabricated by an aerosol cross-linking process for obtaining homogeneous cell distribution: fabrication, seeding efficiency, and cell proliferation and distribution," *Tissue Engineering Part C: Methods*, vol. 19, pp. 784-793, Oct 2013.
- [154] I. T. Ozbolat, H. Chen, and Y. Yu, "Development of 'Multi-arm Bioprinter' for hybrid biofabrication of tissue engineering constructs," *Robotics and Computer-Integrated Manufacturing*, vol. 30, pp. 295-304, 2014.
- [155] J.-H. Shim, J.-S. Lee, J. Y. Kim, and D.-W. Cho, "Bioprinting of a mechanically enhanced three-dimensional dual cell-laden construct for osteochondral

tissue engineering using a multi-head tissue/organ building system," *Journal of Micromechanics and Microengineering*, vol. 22, p. 085014, 2012.

- [156] J. E. Snyder, Q. Hamid, C. Wang, R. Chang, K. Emami, H. Wu, *et al.*, "Bioprinting cell-laden matrigel for radioprotection study of liver by pro-drug conversion in a dual-tissue microfluidic chip," *Biofabrication*, vol. 3, p. 034112, Sep 2011.
- [157] X. H. Wang, Y. N. Yan, Y. Q. Pan, Z. Xiong, H. X. Liu, B. Cheng, *et al.*, "Generation of three-dimensional hepatocyte/gelatin structures with rapid prototyping system," *Tissue Engineering*, vol. 12, pp. 83-90, Jan 2006.
- [158] A. Skardal, J. Zhang, and G. D. Prestwich, "Bioprinting vessel-like constructs using hyaluronan hydrogels crosslinked with tetrahedral polyethylene glycol tetracrylates," *Biomaterials*, vol. 31, pp. 6173-6181, Aug 2010.
- [159] J. Visser, B. Peters, T. J. Burger, J. Boomstra, W. J. Dhert, F. P. Melchels, *et al.*, "Biofabrication of multi-material anatomically shaped tissue constructs," *Biofabrication*, vol. 5, p. 035007, Sep 2013.
- [160] W. Lee, V. Lee, S. Polio, K. Fischer, J.-H. Lee, J. Park, *et al.*, "Three-dimensional cell-hydrogel printer using electromechanical microvalve for tissue engineering," in *Solid-State Sensors, Actuators and Microsystems Conference, 2009. TRANSDUCERS 2009. International*, 2009, pp. 2230-2233.
- [161] Y. Nahmias and D. J. Odde, "Micropatterning of living cells by laser-guided direct writing: application to fabrication of hepatic endothelial sinusoid-like structures," *Nature Protocols*, vol. 1, pp. 2288-2296, 2006 2006.
- [162] P. Soman, P. H. Chung, A. P. Zhang, and S. C. Chen, "Digital Microfabrication of User-Defined 3D Microstructures in Cell-Laden Hydrogels," *Biotechnology and Bioengineering*, vol. 110, pp. 3038-3047, Nov 2013.
- [163] N. E. Fedorovich, M. H. Oudshoorn, D. van Geemen, W. E. Hennink, J. Alblas, and W. J. A. Dhert, "The effect of photopolymerization on stem cells embedded in hydrogels," *Biomaterials*, vol. 30, pp. 344-353, 1// 2009.
- [164] S. Moon, S. K. Hasan, Y. S. Song, F. Xu, H. O. Keles, F. Manzur, *et al.*, "Layer by layer three-dimensional tissue epitaxy by cell-laden hydrogel droplets," *Tissue Engineering Part C: Methods*, vol. 16, pp. 157-166, 2009.
- [165] A. Skardal, D. Mack, E. Kapetanovic, A. Atala, J. D. Jackson, J. Yoo, *et al.*, "Bioprinted Amniotic Fluid-Derived Stem Cells Accelerate Healing of Large Skin Wounds," *Stem Cells Transl. Med.*, vol. 1, pp. 792-802, 10/29 2012.
- [166] D. Houzhu, T. Filippou, and C. C. Robert, "Bioprinting multidimensional constructs: a quantitative approach to understanding printed cell density

and redistribution phenomena," *Biomedical Physics & Engineering Express*, vol. 3, p. 035016, 2017.

- [167] H. Ding, F. Tournemousis, and R. Chang, "A Methodology for Quantifying Cell Density and Distribution in Multidimensional Bioprinted Gelatin-Alginate Constructs," in *12th ASME International Manufacturing Science and Engineering Conference*, Los Angeles, CA, 2017.
- [168] K. Markstedt, A. Mantas, I. Tournier, H. Martínez Ávila, D. Hägg, and P. Gatenholm, "3D Bioprinting Human Chondrocytes with Nanocellulose-Alginate Bioink for Cartilage Tissue Engineering Applications," *Biomacromolecules*, vol. 16, pp. 1489-1496, 2015/05/11 2015.
- [169] D. Tuğrul Tolga, I. Gülseren, and G. Menemşe, "A bioprintable form of chitosan hydrogel for bone tissue engineering," *Biofabrication*, vol. 9, p. 035003, 2017.
- [170] M. T. Poldervaart, B. Goversen, M. de Ruijter, A. Abbadessa, F. P. W. Melchels, F. C. Oner, *et al.*, "3D bioprinting of methacrylated hyaluronic acid (MeHA) hydrogel with intrinsic osteogenicity," *Plos One*, vol. 12, p. e0177628, Jun 2017.
- [171] M. J. Sawkins, B. N. Brown, L. J. Bonassar, F. Rose, and K. M. Shakesheff, "Bioplotting of novel scaffold materials and complex constructs for osteochondral tissue engineering," presented at the Annual meeting of the Tissue and Cell Engineering Society, University of Liverpool, 2012.
- [172] S. Wüst, R. Müller, and S. Hofmann, "3D Bioprinting of complex channels—Effects of material, orientation, geometry, and cell embedding," *Journal of Biomedical Materials Research Part A*, vol. 103, pp. 2558-2570, 2015.
- [173] M. Michael, B. Jana, S. Matthias, and Z.-W. Marcy, "Nanostructured Pluronic hydrogels as bioinks for 3D bioprinting," *Biofabrication*, vol. 7, p. 035006, 2015.
- [174] W. Liu, M. A. Heinrich, Y. Zhou, A. Akpek, N. Hu, X. Liu, *et al.*, "Extrusion Bioprinting of Shear - Thinning Gelatin Methacryloyl Bioinks," *Advanced Healthcare Materials*, vol. 6, p. 1601451, 2017.
- [175] Y. J. Choi, T. G. Kim, J. Jeong, H. G. Yi, J. W. Park, W. Hwang, *et al.*, "3D Cell Printing of Functional Skeletal Muscle Constructs Using Skeletal Muscle-Derived Bioink," *Advanced Healthcare Materials*, vol. 5, pp. 2636-2645, Oct 2016.

- [176] F. Pati, J. Jang, D.-H. Ha, S. Won Kim, J.-W. Rhie, J.-H. Shim, *et al.*, "Printing three-dimensional tissue analogues with decellularized extracellular matrix bioink," *Nature Communications*, vol. 5, p. 3935, 06/02/online 2014.
- [177] W. Schuurman, P. A. Levett, M. W. Pot, P. R. van Weeren, W. J. A. Dhert, D. W. Hutmacher, *et al.*, "Gelatin-Methacrylamide Hydrogels as Potential Biomaterials for Fabrication of Tissue-Engineered Cartilage Constructs," *Macromolecular Bioscience*, vol. 13, pp. 551-561, 2013.
- [178] J. W. Nichol, S. T. Koshy, H. Bae, C. M. Hwang, S. Yamanlar, and A. Khademhosseini, "Cell-laden microengineered gelatin methacrylate hydrogels," *Biomaterials*, vol. 31, pp. 5536-5544, Jul 2010.
- [179] J. A. Benton, C. A. DeForest, V. Vivekanandan, and K. S. Anseth, "Photocrosslinking of Gelatin Macromers to Synthesize Porous Hydrogels That Promote Valvular Interstitial Cell Function," *Tissue Engineering Part A*, vol. 15, pp. 3221-3230, Nov 2009.
- [180] T. Jungst, W. Smolan, K. Schacht, T. Scheibel, and J. Groll, "Strategies and Molecular Design Criteria for 3D Printable Hydrogels," *Chemical Reviews*, vol. 116, pp. 1496-1539, 2015/10/23 2015.
- [181] C. Li, A. Faulkner-Jones, A. R. Dun, J. Jin, P. Chen, Y. Z. Xing, *et al.*, "Rapid Formation of a Supramolecular Polypeptide-DNA Hydrogel for In Situ Three-Dimensional Multilayer Bioprinting," *Angewandte Chemie International Edition*, vol. 54, pp. 3957-3961, Mar 23 2015.
- [182] Y. B. Lee, S. Polio, W. Lee, G. Dai, L. Menon, R. S. Carroll, *et al.*, "Bio-printing of collagen and VEGF-releasing fibrin gel scaffolds for neural stem cell culture," *Experimental Neurology*, vol. 223, pp. 645-652, Jun 2010.
- [183] M. Kesti, C. Eberhardt, G. Pagliccia, D. Kenkel, D. Grande, A. Boss, *et al.*, "Bioprinting Complex Cartilaginous Structures with Clinically Compliant Biomaterials," *Advanced Functional Materials*, vol. 25, pp. 7406-7417, 2015.
- [184] J. Malda, J. Visser, F. P. Melchels, T. Jungst, W. E. Hennink, W. J. Dhert, *et al.*, "25th anniversary article: Engineering hydrogels for biofabrication," *Advanced Materials*, vol. 25, pp. 5011-5028, Sep 25 2013.
- [185] A. Panwar and L. P. Tan, "Current Status of Bioinks for Micro-Extrusion-Based 3D Bioprinting," *Molecules*, vol. 21, p. 685, 2016.
- [186] E. Y. S. Tan and W. Y. Yeong, "Concentric bioprinting of alginate-based tubular constructs using multi-nozzle extrusion-based technique," *International Journal of Bioprinting*, vol. 1, pp. 49-56, 2015.

- [187] M. G. Li, X. Y. Tian, and X. B. Chen, "Modeling of Flow Rate, Pore Size, and Porosity for the Dispensing-Based Tissue Scaffolds Fabrication," *Journal of Manufacturing Science and Engineering*, vol. 131, p. 034501, 2009.
- [188] S. Wüst, M. E. Godla, R. Müller, and S. Hofmann, "Tunable hydrogel composite with two-step processing in combination with innovative hardware upgrade for cell-based three-dimensional bioprinting," *Acta Biomaterialia*, vol. 10, pp. 630-640, 2// 2014.
- [189] S. A. Irvine, A. Agrawal, B. H. Lee, H. Y. Chua, K. Y. Low, B. C. Lau, *et al.*, "Printing cell-laden gelatin constructs by free-form fabrication and enzymatic protein crosslinking," *Biomedical Microdevices*, vol. 17, pp. 1-8, Feb 2015.
- [190] J. M. Lee and W. Y. Yeong, "Understanding the relation between printing and material parameters of time-pressure dispensing system for bioprinting," in *1st International Conference on Progress in Additive Manufacturing*, 2014, p. 459.
- [191] J. M. Lee and W. Y. Yeong, "A preliminary model of time-pressure dispensing system for bioprinting based on printing and material parameters," *Virtual and Physical Prototyping*, vol. 10, pp. 3-8, 2015/01/02 2015.
- [192] Y. Nishiyama, M. Nakamura, C. Henmi, K. Yamaguchi, S. Mochizuki, H. Nakagawa, *et al.*, "Development of a Three-Dimensional Bioprinter: Construction of Cell Supporting Structures Using Hydrogel and State-Of-The-Art Inkjet Technology," *Journal of Biomechanical Engineering*, vol. 131, pp. 035001-035001, 2008.
- [193] Q. Gao, Y. He, J.-z. Fu, A. Liu, and L. Ma, "Coaxial nozzle-assisted 3D bioprinting with built-in microchannels for nutrients delivery," *Biomaterials*, vol. 61, pp. 203-215, 8// 2015.
- [194] Y. S. E. Tan and W. Y. Yeong, "Direct bioprinting of alginate-based tubular constructs using multi-nozzle extrusion-based technique," in *1st International Conference on Progress in Additive Manufacturing*, Singapore, 2014, pp. 423-427.
- [195] D. B. Kolesky, R. L. Truby, A. S. Gladman, T. A. Busbee, K. A. Homan, and J. A. Lewis, "3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs," *Advanced Materials*, vol. 26, pp. 3124-3130, May 21 2014.
- [196] J. S. Miller, K. R. Stevens, M. T. Yang, B. M. Baker, D.-H. T. Nguyen, D. M. Cohen, *et al.*, "Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues," *Nature Materials*, vol. 11, pp. 768-774, 09//print 2012.

- [197] H.-W. Kang, S. J. Lee, I. K. Ko, C. Kengla, J. J. Yoo, and A. Atala, "A 3D bioprinting system to produce human-scale tissue constructs with structural integrity," *Nature Biotechnology*, vol. 34, pp. 312-319, 02/15 2016.
- [198] C. Kucukgul, S. B. Ozler, I. Inci, E. Karakas, S. Irmak, D. Gozuacik, *et al.*, "3D bioprinting of biomimetic aortic vascular constructs with self-supporting cells," *Biotechnology and Bioengineering*, vol. 112, pp. 811-821, 2015.
- [199] T. J. Hinton, Q. Jallerat, R. N. Palchesko, J. H. Park, M. S. Grodzicki, H.-J. Shue, *et al.*, "Three-dimensional printing of complex biological structures by freeform reversible embedding of suspended hydrogels," *Science Advances*, vol. 1, p. e1500758, 2015-10-01 2015.
- [200] W. Wu, A. DeConinck, and J. A. Lewis, "Omnidirectional Printing of 3D Microvascular Networks," *Advanced Materials*, vol. 23, pp. H178-H183, 2011.
- [201] W. Schuurman, V. Khristov, M. W. Pot, P. R. van Weeren, W. J. Dhert, and J. Malda, "Bioprinting of hybrid tissue constructs with tailorable mechanical properties," *Biofabrication*, vol. 3, p. 021001, Jun 2011.
- [202] J. H. Shim, J. Y. Kim, M. Park, J. Park, and D. W. Cho, "Development of a hybrid scaffold with synthetic biomaterials and hydrogel using solid freeform fabrication technology," *Biofabrication*, vol. 3, p. 034102, Sep 2011.
- [203] R. Cornock, S. Beirne, B. Thompson, and G. G. Wallace, "Coaxial additive manufacture of biomaterial composite scaffolds for tissue engineering," *Biofabrication*, vol. 6, p. 025002, Jun 2014.
- [204] A. Blaeser, D. F. D. Campos, U. Puster, W. Richtering, M. M. Stevens, and H. Fischer, "Controlling Shear Stress in 3D Bioprinting is a Key Factor to Balance Printing Resolution and Stem Cell Integrity," *Advanced Healthcare Materials*, vol. 5, pp. 326-333, Feb 2016.
- [205] T. Xu, K. W. Binder, M. Z. Albanna, D. Dice, W. Zhao, J. J. Yoo, *et al.*, "Hybrid printing of mechanically and biologically improved constructs for cartilage tissue engineering applications," *Biofabrication*, vol. 5, p. 015001, Mar 2013.
- [206] R. Gaetani, P. A. Doevendans, C. H. Metz, J. Alblas, E. Messina, A. Giacomello, *et al.*, "Cardiac tissue engineering using tissue printing technology and human cardiac progenitor cells," *Biomaterials*, vol. 33, pp. 1782-1790, Feb 2012.
- [207] K. Zhu, S. R. Shin, T. van Kempen, Y.-C. Li, V. Ponraj, A. Nasajpour, *et al.*, "Gold Nanocomposite Bioink for Printing 3D Cardiac Constructs," *Advanced Functional Materials*, vol. 27, p. 1605352, 2017.

- [208] C. Norotte, F. S. Marga, L. E. Niklason, and G. Forgacs, "Scaffold-free vascular tissue engineering using bioprinting," *Biomaterials*, vol. 30, pp. 5910-5917, Oct 2009.
- [209] R. Gaetani, D. A. M. Feyen, V. Verhage, R. Slaats, E. Messina, K. L. Christman, *et al.*, "Epicardial application of cardiac progenitor cells in a 3D-printed gelatin/hyaluronic acid patch preserves cardiac function after myocardial infarction," *Biomaterials*, vol. 61, pp. 339-348, 2015/08/01/ 2015.
- [210] P. J. Fellows, "Extrusion," in *Food Processing Technology*, P. J. Fellows, Ed., Third ed: Woodhead Publishing, 2009, pp. 456-477.
- [211] C. Muehlenfeld and M. Thommes, "Miniaturization in pharmaceutical extrusion technology: feeding as a challenge of downscaling," *AAPS PharmSciTech*, vol. 13, pp. 94-100, 2012.
- [212] D. L. Cohen, W. Lo, A. Tsavaris, D. Peng, H. Lipson, and L. J. Bonassar, "Increased mixing improves hydrogel homogeneity and quality of three-dimensional printed constructs," *Tissue Engineering Part C: Methods*, vol. 17, pp. 239-248, Feb 2011.
- [213] S. V. Murphy and A. Atala, "3D bioprinting of tissues and organs," *Nature Biotechnology*, vol. 32, pp. 773-785, Aug 5 2014.
- [214] Y. Yu, H. Wen, J. Ma, S. Lykkemark, H. Xu, and J. Qin, "Flexible fabrication of biomimetic bamboo-like hybrid microfibers," *Advanced Materials*, vol. 26, pp. 2494-2499, Apr 23 2014.
- [215] K. Jakab, C. Norotte, F. Marga, K. Murphy, G. Vunjak-Novakovic, and G. Forgacs, "Tissue engineering by self-assembly and bio-printing of living cells," *Biofabrication*, vol. 2, p. 022001, Jun 2010.
- [216] X. B. Chen, "Modeling and control of fluid dispensing processes: a state-of-the-art review," *The International Journal of Advanced Manufacturing Technology*, vol. 43, pp. 276-286, 2008.
- [217] X. B. Chen, "Modeling of Rotary Screw Fluid Dispensing Processes," *Journal of Electronic Packaging*, vol. 129, p. 172, 2007.
- [218] X. B. Chen, G. Shoenau, and W. J. Zhang, "Modeling of time-pressure fluid dispensing processes," *IEEE Transactions on Electronics Packaging Manufacturing*, vol. 23, pp. 300-305, 2000.
- [219] X. B. Chen and K. Jun, "Modeling of positive-displacement fluid dispensing processes," *IEEE Transactions on Electronics Packaging Manufacturing*, vol. 27, pp. 157-163, 2004.

- [220] K. H. Kang, L. A. Hockaday, and J. T. Butcher, "Quantitative optimization of solid freeform deposition of aqueous hydrogels," *Biofabrication*, vol. 5, p. 035001, Sep 2013.
- [221] X. Wang, K. He, and W. Zhang, "Optimizing the fabrication processes for manufacturing a hybrid hierarchical polyurethane-cell/hydrogel construct," *Journal of Bioactive and Compatible Polymers*, vol. 28, pp. 303-319, 2013.
- [222] M. Lovett, K. Lee, A. Edwards, and D. L. Kaplan, "Vascularization Strategies for Tissue Engineering," *Tissue Engineering Part B: Reviews*, vol. 15, pp. 353-370, Sep 2009.
- [223] T. F. Tadros, *Rheology of dispersions : principles and applications / Tharwat F. Tadros*: Weinheim : Wiley-VCH, c2010., 2010.
- [224] A. Tirella, F. Vozzi, C. De Maria, G. Vozzi, T. Sandri, D. Sassano, *et al.*, "Substrate stiffness influences high resolution printing of living cells with an ink-jet system," *Journal of Bioscience and Bioengineering*, vol. 112, pp. 79-85, Jul 2011.
- [225] S. Aktas, D. M. Kalyon, B. M. Marin-Santibanez, and J. Perez-Gonzalez, "Shear viscosity and wall slip behavior of a viscoplastic hydrogel," *Journal of Rheology*, vol. 58, pp. 513-535, Mar 2014.
- [226] I. Leibacher, W. Dietze, P. Hahn, J. Wang, S. Schmitt, and J. Dual, "Acoustophoresis of hollow and core-shell particles in two-dimensional resonance modes," *Microfluidics and Nanofluidics*, vol. 16, pp. 513-524, 2014/03/01 2014.
- [227] D. Vader, A. Kabla, D. Weitz, and L. Mahadevan, "Strain-Induced Alignment in Collagen Gels," *Plos One*, vol. 4, p. e5902, 2009.
- [228] N. de Jonge, F. W. Kanters, F. T. Baaijens, and C. C. Bouten, "Strain-induced Collagen Organization at the Micro-level in Fibrin-based Engineered Tissue Constructs," *Annals of Biomedical Engineering*, vol. 41, pp. 763-774, 2013/04/01 2013.
- [229] S. Hinds, W. Bian, R. G. Dennis, and N. Bursac, "The role of extracellular matrix composition in structure and function of bioengineered skeletal muscle," *Biomaterials*, vol. 32, pp. 3575-3583, 2011/05/01/ 2011.
- [230] R. Chang and W. Sun, "Effects of dispensing pressure and nozzle diameter on cell survival from solid freeform fabrication-based direct cell writing," *Tissue Engineering Part A*, vol. 14, pp. 41-48, Jan 2008.

- [231] Y. Zhao, Y. Li, S. S. Mao, W. Sun, and R. Yao, "The influence of printing parameters on cell survival rate and printability in microextrusion-based 3D cell printing technology," *Biofabrication*, vol. 7, p. 045002, Dec 2015.
- [232] Y. He, F. Yang, H. Zhao, Q. Gao, B. Xia, and J. Fu, "Research on the printability of hydrogels in 3D bioprinting," *Scientific Reports*, vol. 6, p. 29977, 2016.
- [233] Z. Wu, X. Su, Y. Xu, B. Kong, W. Sun, and S. Mi, "Bioprinting three-dimensional cell-laden tissue constructs with controllable degradation," *Scientific Reports*, vol. 6, p. 24474, 04/19/online 2016.
- [234] O. Liliang, Y. Rui, Z. Yu, and S. Wei, "Effect of bioink properties on printability and cell viability for 3D bioplotting of embryonic stem cells," *Biofabrication*, vol. 8, p. 035020, 2016.
- [235] X. T. Shi, S. Ostrovidov, Y. H. Zhao, X. B. Liang, M. Kasuya, K. Kurihara, *et al.*, "Microfluidic Spinning of Cell-Responsive Grooved Microfibers," *Advanced Functional Materials*, vol. 25, pp. 2250-2259, Apr 15 2015.
- [236] G. Camci-Unal, D. Cuttica, N. Annabi, D. Demarchi, and A. Khademhosseini, "Synthesis and Characterization of Hybrid Hyaluronic Acid-Gelatin Hydrogels," *Biomacromolecules*, vol. 14, pp. 1085-1092, 03/28 2013.
- [237] S. T. Koshy, T. C. Ferrante, S. A. Lewin, and D. J. Mooney, "Injectable, porous, and cell-responsive gelatin cryogels," *Biomaterials*, vol. 35, pp. 2477-2487, 12/15 2014.
- [238] J. L. Drury and D. J. Mooney, "Hydrogels for tissue engineering: scaffold design variables and applications," *Biomaterials*, vol. 24, pp. 4337-4351, Nov 2003.
- [239] S. A. Bencherif, A. Srinivasan, F. Horkay, J. O. Hollinger, K. Matyjaszewski, and N. R. Washburn, "Influence of the degree of methacrylation on hyaluronic acid hydrogels properties," *Biomaterials*, vol. 29, pp. 1739-1749, 2008/04/01/ 2008.
- [240] L. A. Hockaday, K. H. Kang, N. W. Colangelo, P. Y. C. Cheung, B. Duan, E. Malone, *et al.*, "Rapid 3D printing of anatomically accurate and mechanically heterogeneous aortic valve hydrogel scaffolds," *Biofabrication*, vol. 4, p. 035005, 2012.
- [241] K. Yue, G. Trujillo-de Santiago, M. M. Alvarez, A. Tamayol, N. Annabi, and A. Khademhosseini, "Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels," *Biomaterials*, vol. 73, pp. 254-271, 12// 2015.

- [242] M. Costantini, S. Testa, E. Fornetti, A. Barbetta, M. Trombetta, S. M. Cannata, *et al.*, "Engineering Muscle Networks in 3D Gelatin Methacryloyl Hydrogels: Influence of Mechanical Stiffness and Geometrical Confinement," *Frontiers in Bioengineering and Biotechnology*, vol. 5, p. 22, 2017.
- [243] J. K. Carrow, P. Kerativitayanan, M. K. Jaiswal, G. Lokhande, and A. K. Gaharwar, "Polymers for Bioprinting," in *Essentials of 3D Biofabrication and Translation*, A. Y. Atala, James J., Ed., ed Boston: Academic Press, 2015, pp. 229-248.
- [244] J. M. Lee and W. Y. Yeong, "Design and Printing Strategies in 3D Bioprinting of Cell-Hydrogels: A Review," *Advanced Healthcare Materials*, vol. 5, pp. 2856-2865, 2016.
- [245] R. G. Wells, "The role of matrix stiffness in regulating cell behavior," *Hepatology*, vol. 47, pp. 1394-1400, Apr 2008.
- [246] B. N. Mason, J. P. Califano, and C. A. Reinhart-King, "Matrix Stiffness: A Regulator of Cellular Behavior and Tissue Formation," in *Engineering Biomaterials for Regenerative Medicine: Novel Technologies for Clinical Applications*, S. K. Bhatia, Ed., ed New York, NY: Springer New York, 2012, pp. 19-37.
- [247] M. T. Poldervaart, H. Gremmels, K. van Deventer, J. O. Fledderus, F. C. Öner, M. C. Verhaar, *et al.*, "Prolonged presence of VEGF promotes vascularization in 3D bioprinted scaffolds with defined architecture," *Journal of Controlled Release*, vol. 184, pp. 58-66, 2014/06/28/ 2014.
- [248] N. E. Fedorovich, W. Schuurman, H. M. Wijnberg, H. J. Prins, P. R. van Weeren, J. Malda, *et al.*, "Biofabrication of osteochondral tissue equivalents by printing topologically defined, cell-laden hydrogel scaffolds," *Tissue Engineering Part C: Methods* vol. 18, pp. 33-44, Jan 2012.
- [249] L. H. Kang, P. A. Armstrong, L. J. Lee, B. Duan, K. H. Kang, and J. T. Butcher, "Optimizing Photo-Encapsulation Viability of Heart Valve Cell Types in 3D Printable Composite Hydrogels," *Annals of Biomedical Engineering*, vol. 45, pp. 360-377, February 01 2017.
- [250] M. Kesti, M. Müller, J. Becher, M. Schnabelrauch, M. D'Este, D. Eglin, *et al.*, "A versatile bioink for three-dimensional printing of cellular scaffolds based on thermally and photo-triggered tandem gelation," *Acta Biomaterialia*, vol. 11, pp. 162-172, 1/1/ 2015.
- [251] C. Colosi, S. R. Shin, V. Manoharan, S. Massa, M. Costantini, A. Barbetta, *et al.*, "Microfluidic Bioprinting of Heterogeneous 3D Tissue Constructs Using Low-Viscosity Bioink," *Advanced Materials*, vol. 28, pp. 677-684, 2016.

- [252] J. M. Lee, M. Zhang, and W. Y. Yeong, "Characterization and evaluation of 3D printed microfluidic chip for cell processing," *Microfluidics and Nanofluidics*, vol. 20, p. 5, January 05 2016.
- [253] W. L. Ng, W. Y. Yeong, and M. W. Naing, "Potential of Bioprinted Films for Skin Tissue Engineering," in *1st International Conference on Progress in Additive Manufacturing*, Singapore, 2014, pp. 441-446.
- [254] W. L. Ng, W. Y. Yeong, and M. W. Naing, "Polyelectrolyte gelatin-chitosan hydrogel optimized for 3D bioprinting in skin tissue engineering," *International Journal of Bioprinting*, vol. 2, pp. 53-62, 2016-01-05 2016.
- [255] S. J. Lee, M. Nowicki, B. Harris, and L. G. Zhang, "Fabrication of a Highly Aligned Neural Scaffold via a Table Top Stereolithography 3D Printing and Electrospinning," *Tissue Engineering Part A*, vol. 23, pp. 491-502, Jun 2017.
- [256] T. K. Merceron, M. Burt, Y. J. Seol, H. W. Kang, S. J. Lee, J. J. Yoo, *et al.*, "A 3D bioprinted complex structure for engineering the muscle-tendon unit," *Biofabrication*, vol. 7, p. 035003, Jun 17 2015.
- [257] M. Costantini, S. Testa, P. Mozetic, A. Barbetta, C. Fuoco, E. Fornetti, *et al.*, "Microfluidic-enhanced 3D bioprinting of aligned myoblast-laden hydrogels leads to functionally organized myofibers in vitro and in vivo," *Biomaterials*, vol. 131, pp. 98-110, Jul 2017.
- [258] A. Boudaoud, A. Burian, D. Borowska-Wykręt, M. Uyttewaal, R. Wrzalik, D. Kwiatkowska, *et al.*, "FibrilTool, an ImageJ plug-in to quantify fibrillar structures in raw microscopy images," *Nature Protocols*, vol. 9, pp. 457-463, 02//print 2014.
- [259] R. Raman, C. Cvetkovic, and R. Bashir, "A modular approach to the design, fabrication, and characterization of muscle-powered biological machines," *Nature Protocols*, vol. 12, pp. 519-533, 03//print 2017.
- [260] E. Gioffredi, M. Boffito, S. Calzone, S. M. Giannitelli, A. Rainer, M. Trombetta, *et al.*, "Pluronic F127 Hydrogel Characterization and Biofabrication in Cellularized Constructs for Tissue Engineering Applications," *Procedia CIRP*, vol. 49, pp. 125-132, 2016/01/01/ 2016.
- [261] C. C. Chang, E. D. Boland, S. K. Williams, and J. B. Hoying, "Direct-write bioprinting three-dimensional biohybrid systems for future regenerative therapies," *Journal of Biomedical Materials Research Part B-Applied Biomaterials*, vol. 98B, pp. 160-170, Jul 2011.
- [262] M.-Y. Yeh, J.-Y. Zhao, Y.-R. Hsieh, J.-H. Lin, F.-Y. Chen, R. D. Chakravarthy, *et al.*, "Reverse thermo-responsive hydrogels prepared from Pluronic F127

- and gelatin composite materials," *RSC Advances*, vol. 7, pp. 21252-21257, 2017.
- [263] D. H. Kim, S.-J. Heo, J.-W. Shin, C. W. Mun, K. M. Park, K. D. Park, *et al.*, "Preparation of thermosensitive gelatin-pluronic copolymer for cartilage tissue engineering," *Macromolecular Research*, vol. 18, pp. 387-391, April 01 2010.
 - [264] D. Tatini, P. Tempesti, F. Ridi, E. Fratini, M. Bonini, and P. Baglioni, "Pluronic/gelatin composites for controlled release of actives," *Colloids and Surfaces B: Biointerfaces*, vol. 135, pp. 400-407, 2015/11/01/ 2015.
 - [265] L. Horváth, Y. Umehara, C. Jud, F. Blank, A. Petri-Fink, and B. Rothen-Rutishauser, "Engineering an in vitro air-blood barrier by 3D bioprinting," *Scientific Reports*, vol. 5, p. 7974, 01/22/online 2015.
 - [266] H. Gaskell, P. Sharma, H. E. Colley, C. Murdoch, D. P. Williams, and S. D. Webb, "Characterization of a functional C3A liver spheroid model," *Toxicology Research*, vol. 5, pp. 1053-1065, 04/27 2016.
 - [267] Y. S. Zhang, A. Arneri, S. Bersini, S.-R. Shin, K. Zhu, Z. Goli-Malekabadi, *et al.*, "Bioprinting 3D microfibrinous scaffolds for engineering endothelialized myocardium and heart-on-a-chip," *Biomaterials*, vol. 110, pp. 45-59, 12// 2016.
 - [268] E. Kang, G. S. Jeong, Y. Y. Choi, K. H. Lee, A. Khademhosseini, and S. H. Lee, "Digitally tunable physicochemical coding of material composition and topography in continuous microfibres," *Nature Materials*, vol. 10, pp. 877-883, Nov 2011.
 - [269] M. Bezanilla, A. S. Gladfelter, D. R. Kovar, and W. L. Lee, "Cytoskeletal dynamics: A view from the membrane," *Journal of Cell Biology*, vol. 209, pp. 329-337, May 2015.
 - [270] B. S. Tyler Novak, Celina M. Twitchell, Corrinus C. van Donkelaar, Sherry L. Voytik-Harbin, and Corey P. Neu, "Mechanisms and Microenvironment Investigation of Cellularized High Density Gradient Collagen Matrices via Densification," *Advanced Functional Materials*, vol. 26, pp. 2617-2628, 2016.
 - [271] M. H. G. Grahame A. Busby, Simon P. MacKay, Philip E. Riches, "Confined compression of collagen hydrogels," *Journal of Biomechanics*, vol. 46, pp. 837-840, 2013.
 - [272] P. Mozetic, S. M. Giannitelli, M. Gori, M. Trombetta, and A. Rainer, "Engineering muscle cell alignment through 3D bioprinting," *Journal of Biomedical Materials Research Part A*, vol. 105, pp. 2582-2588, 2017.

- [273] B. Trappmann, B. M. Baker, W. J. Polacheck, C. K. Choi, J. A. Burdick, and C. S. Chen, "Matrix degradability controls multicellularity of 3D cell migration," *Nature Communications*, vol. 8, p. 371, 2017/08/29 2017.
- [274] S. Khetan, M. Guvendiren, W. R. Legant, D. M. Cohen, C. S. Chen, and J. A. Burdick, "Degradation-mediated cellular traction directs stem cell fate in covalently crosslinked three-dimensional hydrogels," *Nature Materials*, vol. 12, pp. 458-465, May 2013.
- [275] K. M. Schultz, K. A. Kyburz, and K. S. Anseth, "Measuring dynamic cell-material interactions and remodeling during 3D human mesenchymal stem cell migration in hydrogels," *Proceedings of the National Academy of Sciences*, vol. 112, pp. E3757-E3764, July 21, 2015 2015.
- [276] A. Aung, J. Theprungsirikul, H. L. Lim, and S. Varghese, "Chemotaxis-driven assembly of endothelial barrier in a tumor-on-a-chip platform," *Lab on a Chip*, vol. 16, pp. 1886-1898, 2016.
- [277] M. Junkin, S. L. Leung, S. Whitman, C. C. Gregorio, and P. K. Wong, "Cellular self-organization by autocatalytic alignment feedback," *Journal of Cell Science*, vol. 124, pp. 4213-4220, Dec 15 2011.
- [278] V. Hosseini, S. Ahadian, S. Ostrovidov, G. Camci-Unal, S. Chen, H. Kaji, *et al.*, "Engineered Contractile Skeletal Muscle Tissue on a Microgrooved Methacrylated Gelatin Substrate," *Tissue Engineering Part A*, vol. 18, pp. 2453-2465, 10/24 2012.
- [279] N. A. Peppas, J. Z. Hilt, A. Khademhosseini, and R. Langer, "Hydrogels in biology and medicine: from molecular principles to bionanotechnology," *Advanced Materials*, vol. 18, pp. 1345-1360, 2006.
- [280] Y. L. Yap and W. Y. Yeong, "Shape recovery effect of 3D printed polymeric honeycomb," *Virtual and Physical Prototyping*, vol. 10, pp. 91-99, 2015.
- [281] V. Francis and P. K. Jain, "Experimental investigations on fused deposition modelling of polymer-layered silicate nanocomposite," *Virtual and Physical Prototyping*, vol. 11, pp. 109-121, 2016.
- [282] M. X. Gan and C. H. Wong, "Practical support structures for selective laser melting," *Journal of Materials Processing Technology*, vol. 238, pp. 474-484, 12// 2016.
- [283] N. Meisel and C. Williams, "An Investigation of Key Design for Additive Manufacturing Constraints in Multimaterial Three-Dimensional Printing," *Journal of Mechanical Design*, vol. 137, p. 111406, 2015.

- [284] L. M. B. Rodgers, "Extrusion-based additive manufacturing process with part annealing," ed: Google Patents, 2012.
- [285] E. Barnett and C. Gosselin, "Weak support material techniques for alternative additive manufacturing materials," *Additive Manufacturing*, vol. 8, pp. 95-104, 10// 2015.
- [286] B. Duan, E. Kapetanovic, L. A. Hockaday, and J. T. Butcher, "Three-dimensional printed trileaflet valve conduits using biological hydrogels and human valve interstitial cells," *Acta Biomaterialia*, vol. 10, pp. 1836-1846, May 2014.
- [287] K. Arai, S. Iwanaga, H. Toda, C. Genci, Y. Nishiyama, and M. Nakamura, "Three-dimensional inkjet biofabrication based on designed images," *Biofabrication*, vol. 3, p. 034113, Sep 2011.
- [288] J. S. Lee, J. M. Hong, J. W. Jung, J. H. Shim, J. H. Oh, and D. W. Cho, "3D printing of composite tissue with complex shape applied to ear regeneration," *Biofabrication*, vol. 6, p. 024103, Jun 2014.
- [289] G. S. Fiorini and D. T. Chiu, "Disposable microfluidic devices: fabrication, function, and application," *BioTechniques*, vol. 38, pp. 429-446, 2005.
- [290] A. J. Capel, S. Edmondson, S. D. Christie, R. D. Goodridge, R. J. Bibb, and M. Thurstans, "Design and additive manufacture for flow chemistry," *Lab on a Chip*, vol. 13, pp. 4583-4590, 2013.
- [291] S. Zhao, H. Cong, and T. Pan, "Direct projection on dry-film photoresist (DP 2): do-it-yourself three-dimensional polymer microfluidics," *Lab on a Chip*, vol. 9, pp. 1128-1132, 2009.
- [292] J. R. Anderson, D. T. Chiu, R. J. Jackman, O. Cherniavskaya, J. C. McDonald, H. Wu, *et al.*, "Fabrication of topologically complex three-dimensional microfluidic systems in PDMS by rapid prototyping," *Analytical Chemistry*, vol. 72, pp. 3158-3164, 2000.
- [293] Y. Liao, J. Song, E. Li, Y. Luo, Y. Shen, D. Chen, *et al.*, "Rapid prototyping of three-dimensional microfluidic mixers in glass by femtosecond laser direct writing," *Lab on a Chip*, vol. 12, pp. 746-749, 2012.
- [294] J. C. McDonald, M. L. Chabinyc, S. J. Metallo, J. R. Anderson, A. D. Stroock, and G. M. Whitesides, "Prototyping of microfluidic devices in poly(dimethylsiloxane) using solid-object printing," *Analytical Chemistry*, vol. 74, pp. 1537-1545, 2002.
- [295] J. Moore, A. McCuiston, I. Mittendorf, R. Ottway, and R. D. Johnson, "Behavior of capillary valves in centrifugal microfluidic devices prepared by three-

dimensional printing," *Microfluidics and Nanofluidics*, vol. 10, pp. 877-888, 2011/04/01 2011.

- [296] A. I. Shallan, P. Smejkal, M. Corban, R. M. Guijt, and M. C. Breadmore, "Cost-effective three-dimensional printing of visibly transparent microchips within minutes," *Analytical Chemistry*, vol. 86, pp. 3124-3130, 2014.
- [297] O. H. Paydar, C. N. Paredes, Y. Hwang, J. Paz, N. B. Shah, and R. N. Candler, "Characterization of 3D-printed microfluidic chip interconnects with integrated O-rings," *Sensors and Actuators A: Physical*, vol. 205, pp. 199-203, 2014.
- [298] C.-M. Ho and Y.-C. Tai, "Micro-electro-mechanical-systems (MEMS) and fluid flows," *Annual Review of Fluid Mechanics*, vol. 30, pp. 579-612, 1998.
- [299] X. Cui, D. Dean, Z. M. Ruggeri, and T. Boland, "Cell damage evaluation of thermal inkjet printed Chinese hamster ovary cells," *Biotechnology and Bioengineering*, vol. 106, pp. 963-969, 2010.
- [300] W. Schrott, Z. Slouka, P. Červenka, J. Ston, M. Nebyla, M. Přibyl, *et al.*, "Study on surface properties of PDMS microfluidic chips treated with albumin," *Biomicrofluidics*, vol. 3, p. 044101, 2009.
- [301] Y. Huang, S. Liu, W. Yang, and C. Yu, "Surface roughness analysis and improvement of PMMA-based microfluidic chip chambers by CO₂ laser cutting," *Applied Surface Science*, vol. 256, pp. 1675-1678, 2010.
- [302] R. Singh, "Process capability study of polyjet printing for plastic components," *Journal of Mechanical Science and Technology*, vol. 25, pp. 1011-1015, Apr 2011.
- [303] J. C. McDonald, D. C. Duffy, J. R. Anderson, D. T. Chiu, H. Wu, O. J. Schueller, *et al.*, "Fabrication of microfluidic systems in poly (dimethylsiloxane)," *Electrophoresis*, vol. 21, pp. 27-40, 2000.
- [304] S. Okushima, T. Nisisako, T. Torii, and T. Higuchi, "Controlled Production of Monodisperse Double Emulsions by Two-Step Droplet Breakup in Microfluidic Devices," *Langmuir*, vol. 20, pp. 9905-9908, 2004/11/01 2004.
- [305] J. H. Xu, S. W. Li, J. Tan, Y. J. Wang, and G. S. Luo, "Controllable Preparation of Monodisperse O/W and W/O Emulsions in the Same Microfluidic Device," *Langmuir*, vol. 22, pp. 7943-7946, 2006/09/01 2006.
- [306] R. J. Good and M. Koo, "The effect of drop size on contact angle," *Journal of Colloid and Interface Science*, vol. 71, pp. 283-292, 1979.

- [307] A. Marmur, "Equilibrium contact angles: theory and measurement," *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, vol. 116, pp. 55-61, 1996.
- [308] D. Chu, A. Nemoto, and H. Ito, "Effects of geometric parameters for superhydrophobicity of polymer surfaces fabricated by precision tooling machines," *Microsystem Technologies*, vol. 20, pp. 193-200, 2014/02/01 2014.
- [309] Z. Yoshimitsu, A. Nakajima, T. Watanabe, and K. Hashimoto, "Effects of surface structure on the hydrophobicity and sliding behavior of water droplets," *Langmuir*, vol. 18, pp. 5818-5822, 2002.
- [310] I. Mironi-Harpaz, D. Y. Wang, S. Venkatraman, and D. Seliktar, "Photopolymerization of cell-encapsulating hydrogels: Crosslinking efficiency versus cytotoxicity," *Acta Biomaterialia*, vol. 8, pp. 1838-1848, 2012.
- [311] H. Ota, R. Yamamoto, K. Deguchi, Y. Tanaka, Y. Kazoe, Y. Sato, *et al.*, "Three-dimensional spheroid-forming lab-on-a-chip using micro-rotational flow," *Sensors and Actuators B: Chemical*, vol. 147, pp. 359-365, 2010.