Development of novel hydrogel composites for bioprinting and engineering of thick tissues

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**NANYANG
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**DEVELOPMENT OF NOVEL HYDROGEL
COMPOSITES FOR BIOPRINTING AND
ENGINEERING OF THICK TISSUES**

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DEVELOPMENT OF NOVEL HYDROGEL COMPOSITES FOR BIOPRINTING AND ENGINEERING OF THICK TISSUES

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Abstract

Tissue engineering (TE) is a technology that combines life sciences and engineering knowledge to restore and replace the functionality of impaired tissues and organs. However, the research work for tissue engineering has achieved limited success in engineering thick tissues. The goal of this work is to address two issues encountered in current technologies when creating 3D thick tissue structures. The major issue is vascularization or creating a 3D complex perfusable vasculature structure. Recently, an emerging solution is 3D bioprinting, a technology that integrates cells and bioinks (biomaterials) with an automated fabrication system such as a bioprinter. In 3D bioprinting, hydrogel, which is biocompatible with cells, is usually used as a cell carrier or bioink. However, most of hydrogels that have good biocompatibility have low printability or low mechanical strength. To solve this problem, a novel hydrogel composite has been developed which is both biocompatible and bioprintable. Separately, another issue in engineering thick tissues is the convenience in transfer and assembly of individual high cell density constructs such as cell laden hydrogels or cell sheets. In this research, another hydrogel composite, a biodegradable membrane modified with collagen gel has been developed to address this issue.

A large part of this thesis focuses on the synthesis, characterization, 3D printing and modelling of a novel hydrogel composite - Pluronic-Gelatin methacrylate (Plu-GelMA). Pluronic needs to be modified into pluronic monocarboxylate (Plu-MP) before it can physically interact with GelMA. After synthesis, the Plu-GelMA hydrogel composites at different mass ratio are studied. A series of characterization have been performed, including chemical tests (NMR and FTIR),

mechanical tests (compression and tensile test), rheological test, physical tests (morphology and microstructure analysis, water swelling properties, perfusion test and diffusion test), printability test and biological test (live/dead staining, Prestoblue and immunohistology). The results show that at the mass ratio 2:1 of PluMP:GelMA, the composite has the best printability and biocompatibility. Moreover, based on immunohistology results, this hydrogel composite is able to support HUVECs differentiation, evidenced by the expression of two endothelial cell markers. Thus, this hydrogel composite can serve as a new bioink to create 3D complex structures that can mimic vascular branches and could be a potential solution to the vascularization problem.

A second part of this thesis investigates solvent-free membrane fabrication and membrane surface modification for application of an intermediate cell carrier for transfer and assembly of cell-laden hydrogels and cell sheets. Briefly, a layer of polymer particles is directly spread on top of water to form surface suspension. After that, with the use of heat, a solid powder layer transforms into a melted layer and a porous thin membrane can be obtained after cooling. Membrane characterization is separated into two parts. The first part focuses on the physical characteristics of membrane such as thickness, surface roughness, mechanical strength, pore size and porosity. The second part of the experiment investigates chemical properties and surface modification. The L929 *in vitro* cell compatibility study on the modified membrane shows good cell attachment and proliferation.

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List of Symbols

ΔP	Gauge pressure
d	Diameter of printed strand
D	Diameter of the nozzle
L	Length of the nozzle
n	Power law index
η	Apparent viscosity
η_0	Zero viscosity
$\dot{Q}$	Volumetric flow rate
R	Radius of the nozzle
R_a	Surface roughness
T_g	Glass transition temperature
T_m	Melting temperature (melting point)
$\dot{\gamma}$	Shear rate
τ_w	Maximum stress in capillary flow
$\dot{\gamma}_w$	Capillary shear rate
$\dot{v}$	Stage moving speed
W_{swollen}	Hydrogel swollen weight
W_{dry}	Hydrogel dried weight

List of Abbreviations

2D	Two dimension
2PP	Two photon polymerization
3D	Three dimension
AAc	Acrylic acid
AM	Additive manufacturing
ATR-FTIR	Attenuated total reflectance Fourier transform infrared spectroscopy
BMSCs	Bone marrow stromal cells
CAD	Computer aided design
CMC	Critical micelles concentration
Col-I	Collagen type I
CT	Computerized tomography
DMAP	4-Dimethylaminopyridine
DMEM	Dulbecco's Modified Eagle's Medium
DOE	Design of experiment
DPBS	Dulbecco's Phosphate Buffered Saline
DSC	Differential scanning calorimetry
ECM	Extracellular matrix
EDC	N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
FBS	Fetal bovine serum
FDM	Fused deposition modelling
FRESH	Freeform reversible embedding of suspended hydrogels
FTIR	Fourier transform infrared spectroscopy
GelMA	Gelatin methacrylate
H&E	Haematoxylin and eosin
HA	Hyaluronic acid
HAZ	Heat-affected zone
HUVECs	Human umbilical vein endothelial cells
LCST	Lower critical solution temperature
MC	Methyl cellulose
MWCO	Molecular weight cutoff
NHS	N-hydroxysuccinimide
NMR	Nuclear magnetic resonance
NMR	Nuclear magnetic resonance spectroscopy
PBS	Phosphate buffer saline
PCL	Polycaprolactone
PECE	Polyethylene glycol (PEG)-polycaprolactone (PCL)-polyethylene glycol (PEG) block

PEDGA	Polyethylene glycol dimethacrylate
PEG	Polyethylene glycol
PEGDMA	Poly(ethylene glycol) dimethacrylate
PEO	Polyethylene oxide
PLGA	Poly(lactide- co-glycolide)
PLLA	Poly(l-lactic) acid
Plu-GelMA	Pluronic-Gelatin methacrylate
Plu-MP	Pluronic monocarboxylate
Pluronic-DA	Pluronic diacrylate
PNIPAAM	Poly (N-isopropylacrylamide)
PPO	Polypropylene oxide
PVA	Polyvinyl alcohol
RF	Radio frequency
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscope
SLA	Stereolithography apparatus
SPELA	Star poly(ethylene glycol-co-lactide) acrylate
TE	Tissue engineering
TEA	Triethanolamine
UCST	Upper critical solution temperature
UV	Ultra violet
VWF	von Willebrand Factor

Chapter 1 Introduction

1.1. Background

Tissue engineering (TE) is an interdisciplinary field that integrates the principles of engineering and life sciences in order to repair and restore the damaged tissues and organs [1, 2]. It requires a combination and integration of material sciences, biology, chemistry and engineering knowledge. Materials used for tissue engineering applications have to be biocompatible and biodegradable *in vivo*, for their important role of supporting cells forming into tissue structure [3]. TE requires the use of scaffold to support cell attachment. TE scaffold must (1) be porous and have interconnected pores to promote cell migration inside the structure, (2) be able to create biocompatible or bioactive environment to cells, (3) have proper design for pore size and pore configuration to support tissue infiltration, (4) have sufficient mechanical properties to maintain structural integrity and mechanical function during implantation [4] and (5) be able to fully degrade *in vivo* at a desired rate and produce minimal side effect (e.g. toxicity and inflammation) inside the body [5].

However, one serious issue in TE is the lack of vascularization which leads to hypoxia (insufficient oxygen and nutrient). Hypoxia is the main reason that causes non-homogeneity in engineered tissue structure [6]. Nowadays, there are many approaches trying to solve this problem and they can be separated into cell-based approach (e.g. pre-vascularization and *in vitro* neo-angiogenesis) and scaffold-based approach (selection of materials and fabrication techniques) [7]. Although many researches have proposed promising solutions for this problem, vascularization still remains as a main obstacle in TE [6-8].

An integration between additive manufacturing (AM) techniques and biodegradable materials has been used to explore TE applications because it has shown the potential to construct and organize complex tissue structures [9, 10]. However, most scaffold-based AM technologies use solid synthetic polymers and cannot provide a suitable environment for cells due to poor surface characteristics of synthetic polymers. Hence, a new scaffold-free, cell-based AM technique has been introduced in recent years. This breakthrough technology is named “bioprinting”, which has shown promise to produce novel soft scaffold structures [11, 12]. In bioprinting, hydrogel and cells are combined into a mixture for printing. The end product is a gel-like scaffold, which is unlike the solid polymer scaffolds made by conventional AM methods.

Bioprinting started with the idea of combining additive manufacturing (3D printing) with living materials to fabricate complex 3D tissues *in vitro*. Compared to microfabrication technologies such as photolithography [13], silicon wafer fabrication process [14], electrohydrodynamic [15] and combination of each technique [16], bioprinting is yet to achieve a resolution down to nanometer, but it has clear advantages on direct cell fabrication and 3D complex structure production. A bioprinter can directly embed cells inside a hydrogel for fabrication in a single step, but most lithography-based techniques need a few steps to create a pattern of cells [16]. Moreover, lithography technologies are 2D and are more suitable for patterning of flat structures such as micro-fluidic chips, rather than fabricating 3D complex constructs.

To date bioprinting has been used for many applications from drug testing, cancer study, stem cell study and tissue engineering [12, 17-19]. Firstly, bioprinting technology provides high precision control from the robotic system, which can

be used for cell co-culture, cell patterning and fabrication of 3D complex tissue model [17, 18, 20-24]. Secondly, by involving robotic machines and computer aided design (CAD) with biological substrates [25], it is possible to create a complex tissue structure with living cells. Moreover, the use of automated machine can overcome the problems of repeatability and upscaling fabrication as found in conventional TE technologies [17, 26, 27]. Lastly, the bioprinting technique is relatively simple to use and provides rapid tissue model prototypes for researchers to study. As shown in Figure 1.1, three main components of bioprinting technology are:

- (i) living organisms which mainly refer to living cells;
- (ii) bioink which mostly are made from hydrogel or cell culture medium [18, 26, 28]. Growth factors may be added to bioink to promote cell growth and the formation [17, 29] such as connective tissue growth factor [30] or fibroblast promoting growth factor [29]; and
- (iii) bioprinter which is the robotic system that has been used to generate CAD file (from CT scan or design software) and to fabricate the tissue structure [26, 31, 32].

Figure 1.1 shows the relationship of the three components. Since bioink supports the cells during printing, it must be biocompatible and be able to provide sufficient mechanical strength and create a suitable environment for cells to grow [27, 33]. On the other hand, a bioprinter should provide high cell viability and high cell density during and after printing to promote cell proliferation and tissue formation [17, 18, 26]. Finally, hydrogel or bioink which acts as a cell carrier needs to have high printability (flowability) during printing and achieve good resolution for printed construct [26, 34, 35]. However, most hydrogels have low

shape integrity before crosslinking which are difficult to support 3D structure [9, 36]. Thus, new types of bioinks are needed in order to achieve the 3D complex structure for vascularization applications.

Figure 1.1. Summary of essential components and relationship of each component for bioprinting.

Besides complex structures, high cell density is also necessary in order to promote cell proliferation and differentiation. Nowadays, the high cell density can be achieved by using cell sheet technology. Cell sheet technology is unique because it is a bottom-up tissue fabrication technique which differs from conventional TE fabrication approaches [37, 38]. Cell sheet technology does not require the use of a scaffold but only needs cells. Cells are grown into monolayer cell sheets. Thus, this method yields high cell density because it ensures that all the cells are physically connected to one another by integrating with their extracellular matrix (ECM) [37, 39]. Cell sheet technology has advanced many TE applications from cardiac tissues to vascularization [40-43]. However, in cell sheet technology, the use of thermo-responsive polymer is required in order to separate cell sheet layer from the petri dish surface. Normally, a thin layer of poly

(N-isopropylacrylamide) (approximately 20 nm) is coated on the petri dish surface in order to release the cell sheet at lower temperature around 32 °C [40, 44, 45]. Moreover, multiple cell sheet layers have to be manually stacked one by one on the petri dish, hence a plunger with thermo-responsive hydrogel coating is required to pick up one cell sheet from one dish and transfer it to another dish. In other words, the stacking process highly relies on plunger equipment and multiple manual steps [44, 46]. One layer of cell sheet has a thickness of around 10 μm [41] and it requires at least 100 layers of cell sheets to create 1 mm tissue construct. Therefore, it is inefficient to manually detach, transfer and stack the cell sheet layer by layer 100 times. In order to solve this problem, a solvent free biodegradable membrane technique has been proposed as an alternative solution. Thus, creating cell sheet on the PCL membrane will be investigated, in which the membrane is used as a platform to transfer cell sheet in a more efficient, robust and scalable manner.

Membrane, which is a porous thin sheet, is suitable to be used as a TE scaffold, and biodegradable porous membrane has been used for many applications in tissue engineering and biomedical engineering. Over the past decades, there are many reports that show the potential of biodegradable membranes when used as TE scaffolds for different types of tissues such as blood vessel [47], heart valve [48], heart tissue [49], bone [50], skin [51] and muscle [52]. Currently, some common methods that are available to fabricate membrane for TE application are solvent (solution) casting and electrospinning. Solution casting and electrospinning usually require the use of an organic solvent to dissolve scaffold materials [53], thus the solvent cast film and electrospun membrane scaffold are susceptible to contamination with toxic remnants.

Hence, there are virtually no membrane fabrication methods that do not need further processing to create pores or remove solvent residues. Membrane fabrication method that is free of organic solvent with surface modification is another potential solution for successful for high cell density transfer. Thus, by using two approaches which enables a 3D complex structure and high cell density transfer from biodegradable membrane platform, these will provide a solution for vascularization issue in TE.

1.2. Objectives

This research intends to develop novel hydrogel composites to address two issues in creating thick tissues. The major one is bioprinting of a perfusable 3D vasculature-like structure. The minor one is a membrane-based carrier for easy transfer and assembly of high cell density constructs. Therefore, the objectives of this project are as follows:

- To develop a new hydrogel composite that has both biocompatibility and good printability and shape integrity for bioprinting.
- To investigate the characteristics and *in vitro* biocompatibility of the new hydrogel composite.
- To fabricate the 3D complex perfusable hollow structures for vascularization and TE applications.
- To model the behaviour of thermo-responsive hydrogel in extrusion based bioprinter.
- To develop a solvent-free membrane with surface hydrogel modification for cell carrier application, and to study the *in vitro* biocompatibility.

1.3. Scope

The scope of this project is as follows:

- Understanding the thermo-responsive hydrogel for bioprinting.
- Understanding the phenomena on the extrusion bioprinting process.
- Characterizing mechanical, physical, chemical and biological properties of the new hydrogel composite.
- Understanding the gelation mechanism and combination of model-support materials printing process.
- Characterizing and modifying the membrane properties for *in vitro* cell culture study and cell carrier application.

1.4. Organisation of report

This thesis begins with an introduction (Chapter 1) which includes background, objectives and scope of this project.

Chapter 2 covers literature review of the thermo-responsive hydrogel characteristics including sources, gelation mechanism, gelation temperature, gelation concentration and the applications in TE and bioprinting. Additionally, the roles of support materials in bioprinting are also included. This chapter ends with a review on the biodegradable polymer film and membrane fabrication and modification processes.

Chapter 3 presents a mathematical model of bioprinting thermo-responsive hydrogels on an extrusion based bioprinter.

Chapter 4 presents the synthesizing of new hydrogel composite as well as chemical, physical, mechanical and rheological characteristics.

Chapter 5 describes *in vitro* biological studies of the hydrogel composites.

Chapter 6 discusses the results of membrane fabrication and surface modification of membrane.

Chapter 7 concludes current research and proposes future research plans.

Publications resulting from this work are listed as well.

Chapter 2 Literature review

Chapter 2 reviews the literature on two main topics. The first part discusses about the state of art of thermo-responsive hydrogels in bioprinting technology. The need of new bioinks for tissue engineering applications and the roles of support materials are also highlighted in this chapter. The second topic is on biodegradable materials used in film and membrane fabrication and also surface modification techniques.

2.1. Thermo-responsive hydrogels for bioprinting and tissue engineering

Hydrogel is the most common bioink and is a polymeric network that contains either mostly water or other specific biological fluids [54]. There are many types of hydrogels including UV crosslinkable hydrogels and thermo-responsive hydrogels [55]. Thermo-responsive hydrogels are commonly used for injectable TE applications [56-58]. Recently, they have been explored for bioprinting applications due to the fact that (i) it is easy to tune the sol-gel state by changing temperature (rapid gelation), (ii) it is easily extruded or printed with good shape fidelity and (iii) high resolution achievable due to rapid gelation [9]. Moreover, some of them also provide good cell compatibility. In TE, hydrogels need to be biodegradable and provide a suitable environment for cells to grow [59, 60]. In bioprinting, hydrogels need to provide good printability in order to create complex 3D structures that mimic microenvironment, natural shape and vascularization [36, 55]. There are many thermo-responsive hydrogels, but depending on origin, gelation mechanism or polymer chain structure, they can have different mechanical and biological properties. In this section, six groups of thermos-responsive hydrogels that have been successfully bioprinted will be discussed.

2.1.1. Natural thermo-responsive hydrogels

2.1.1.1. Gelatin and derivatives

Gelatin has been used in different types of bioprinters in a range of concentrations (2 - 20 % w/v as shown in Table 2.1), but most of which are pneumatic bioprinters [35, 61-65]. Gelatin is a natural protein (see the chemical structure in Figure 2.1) and can be extracted from many animal sources [66, 67]. Gelatin is derived from collagen, and the structure of gelatin is a helix when it is in gel state and random coils when it is in the solution state [68, 69]. As shown in Figure 2.2a, the gelation mechanism of gelatin undergoes a coil-helix transition, in which random coils transform into helix structures when the temperature is lower than 35 °C (upper critical solution temperature, UCST) and the gelation speed goes faster when the temperature is around 4-25 °C [55, 68-70]. Human body temperature is around 37 °C, hence the *in vitro* bioprinting experiment should be conducted at this temperature. However, gelatin alone is not suitable for bioprinting because it turns into liquid phase at 37 °C [42, 71]. Therefore, gelatin usually undergoes chain modification and chemical crosslinking to enhance mechanical properties to make its shape more stable at 37 °C [59, 72]. One common derivative of gelatin is gelatin methacrylate (GelMA), which is generated from the reaction between methacrylate anhydride and gelatin at around 50 °C under vigorous stirring condition as shown in Figure 2.2b [73, 74]. GelMA can react with many types of photo-initiators such as irgacure and Eosin Y to acquire UV photocuring property while still preserving good cell viability as shown in Figure 2.2c [62, 64, 75]. Even though gelatin alone does not provide good printability at human body temperature, both gelatin and GelMA have been used for

a variety of TE applications from vascularization to cell patterning as shown in Table 2.1 [62, 74, 76-78]. Recently, Hinton *et al.* published “FRESH” technique, in which gelatin was used as a sacrificial material to hold other hydrogel parts. The technique is an advancement on the use of gelatin to create a 3D complex cell laden hydrogel structure [79].

Figure 2.1. Chemical structure of natural based thermos-responsive hydrogels.

Figure 2.2. a) Coil-helix gelation mechanism due to change in temperature, b) Chemical reaction of GelMA synthesis and c) Optical microscope of cell-laden hydrogen and cell viability of crosslinkable gelatin printed structure at day 7 with scale bar of 500 μm (Reproduced with permission, Copyright 2013, Elsevier) [64].

2.1.1.2. Methylcellulose (MC)

Cellulose is the natural polymer found in plant cell walls [70, 80]. Methyl cellulose (MC) is a derivative of cellulose and is water-soluble because of hydroxyl group substitution on some hydrophobic groups in the chain. MC is a polysaccharide-like sugar or starch as shown in Figure 2.1 [68, 81]. Unlike gelatin, the gelation mechanism of methylcellulose is based on hydrophobic interactions, i.e. when the temperature reaches lower critical solution temperature (LCST), the hydrophobic parts in the chain become dominant and link each other to create the gelation network [68, 81, 82]. MC is usually dispensed by using pneumatic extrusion bioprinter at a concentration around 4 % w/v [83]. Due to polysaccharide structure, it does not have

bio-active properties that favour the attachment of adherent cells. However, its unique gelation temperature at 40 – 50 °C is close to human body temperature [68, 81], suggesting that it could be used as support materials during bioprinting. Moreover, integrating other hydrogels with MC for TE applications has also been explored [80, 84].

2.1.1.3. Agarose

Agarose is the main component of agar in company with agaropectin [85]. Agarose is a natural polysaccharide that can be found in cell walls of red algae [69]. As shown in Figure 2.1, agarose consists of two basic saccharide repeating units which are (1,3) linked β -D-galactose (left) and (1,4) linked α -L-3,6-anhydrogalactose (right) [86]. Agarose gelation mechanism is similar to gelatin but it will change from random coils to double helix chains after the temperature reaches LCST (around 30 – 40 °C) [69, 87]. Agarose is a thermo-responsive hydrogel that has been used in both pneumatic- and mechanical- extrusion based bioprinter at low concentration (1 – 5 % w/v) [33, 55, 83]. Instead of printing agarose with cells directly it can be used as a sacrificial material to create a mould or pattern [33, 74]. For example, in the work of Bertassoni *et al.*, agarose strands were printed and used to create the micro-channel for vascularised TE constructs as shown in Figure 2.3a [88]. Agarose can also be mixed with other hydrogels to enhance thermo-responsive properties for soft tissue TE applications [89]. Moreover, agarose hydrogel also shows potential for bone tissue engineering application [83].

Figure 2.3. **a)** Bioprinted agarose template embedded in GelMA. Scale bar: 3mm (Reproduced with permission, Copyright 2014, Royal Society of Chemistry) [88] and **b)** Bioprinted fibrochondrocyte cells seeded in 17.5 mg/ml collagen hydrogel. Scale bar: 50 μ m (Reproduced with permission, Copyright 2016, American Chemical Society) [90].

2.1.1.4. Collagen

Collagen is one of the most abundant natural polymers in mammals and human. About 30 types of collagen have been identified and all are in a form of triple-helix tertiary molecular structure [91, 92]. The most common type of collagen that has been used in bioprinting is collagen type I (Col-I) [55, 93]. Unlike gelatin, which has denatured tertiary structure and can be crosslinked for gelation by a simple change of temperature, collagen is usually prepared in an acidic solution and then changed into solid form (hydrogel) by both neutralization (increase pH) and thermal gelation [36, 55, 92]. Due to a different gelation mechanism, collagen gel takes longer time to gel (at 37 °C) compared to other thermo-responsive hydrogels [36, 93]. Collagen hydrogel is usually used at relatively low concentration 0.1 %w/v to medium concentration around 3 %w/v, which can be printed with both inkjet and extrusion bioprinter or printed via hybrid techniques [55, 90, 94, 95]. In addition, mixing with other hydrogels such as alginate, fibrin and hyaluronic acid can improve printability and stability [36, 55, 96]. Collagen hydrogel and its mixture have been used for many

biomedical applications such as wound healing and cartilage TE (as shown in Figure 2.3b) [90, 93, 97].

2.1.2. Synthetic thermo-responsive hydrogels

2.1.2.1. Pluronic and derivatives

Pluronic is a synthetic block polymer that consists of polyethylene oxide (PEO) and polypropylene oxide (PPO) as a triblock polymer chain shown in Figure 2.4 and 2.5b. PEO is a hydrophilic part while PPO is a hydrophobic part [98, 99]. Many types of pluronics are available, such as F68, F108 and F127, depending on the ratio between PEO and PPO (n = no. of PEO and m = no. of PPO in pluronic chain). However, the most common pluronic that has been used with bioprinter is pluronic F127 due to stability of viscosity at room temperature. [55, 62, 83, 98]. Pluronic gelation mechanism is an interaction of micelles, which relies on both concentration and temperature as shown in Figure 2.5. Firstly, when pluronic concentration is lower than critical micelles concentration (cmc), only single chains are present in the solution. After the concentration is increased, pluronic chains start to aggregate. At cmc, it will form micelles by connecting the hydrophobic parts together (Figure 2.5a). Once micelles are formed, the change in temperature makes pluronic micelles pack together (as shown in Figure 2.5c) and shift from solution into gel phase [68, 70, 98].

Pluronic F127 (Lutrol F127) can be printed with both extrusion based and valve based bioprinter [55, 83, 100]. Because of cmc, pluronic concentration has to be at least 20% w/v to be able to form a gel and commonly printed at 25-40 %w/v [68, 101]. The gelation temperature for pluronic F127 is around 10 – 40 °C, which is suitable for printing at both room temperature and human body temperature (Figure 2.6a) [68, 102].

Figure 2.4. Chemical structure of synthetic based thermo-responsive hydrogels.

Figure 2.5. a) Pluronic micelles gelation mechanism due to change in temperature, b) Pluronic chain structure and c) Micelles packing.

Pluronic F127 shows good printability as shown in Figure 2.6b and 2.6c. However, it is not bio-active and not suitable for long term cell viability [83]. Therefore, it is usually used as sacrificial material to create a track or a mould for indirect printing [103, 104]. For example, Kolesky *et al.* used pluronic to create the track to dispense GelMA for micro-channel printing, which is similar to schematic shown in Figure 2.6d [62].

Figure 2.6. a) Pluronic becomes solid when the temperature is changed to 37 °C; b) CAD drawing for bioprinting; c) Printed hydrogel part using pluronic F127; and d) Pluronic mould with UV-crosslinked hydrogel printing strategy.

Pluronic F127 has a very good solubility in water (up to 40 wt%) [55] especially at 4 °C, therefore, it is possible to remove the guide track or pattern completely by putting it in an ice bath or fridge and washing with cold water [54, 104]. Lastly, pluronic has poor mechanical properties due to micelles gelation. Chain modification (e.g. pluronic diacrylate, pluronic-DA) or combining with other hydrogels such as collagen or gelatin, is usually necessary in order to enhance mechanical properties and cell compatibility [100, 105, 106]

2.1.2.2. PEG (PEO) based block polymer

PEO or PEG can form not only pluronics but also other types of block polymer with thermo-responsive properties. There are a few other types of PEG block polymer that have been used for biomedical applications, such as polyethylene glycol (PEG)-polycaprolactone (PCL)- polyethylene glycol (PEG) block, PECE and poly(D,L-lactide-co-glycolide) - poly(ethylene glycol) - poly(D,L-lactide- co-glycolide) triblock copolymers (PLGA-PEG-PLGA), (structures are shown in Figure 2.4) [80, 107, 108]. PECE and PLGA-PEG-PLGA are commonly used as injectable hydrogel for tissue engineering and drug delivery [80, 109]. Due to the presence of PEG, the gelation mechanism of these hydrogels involves both micelle interaction and hydrophobic interaction as shown in Figure 2.7. First, the block polymer forms micelles by looping hydrophobic and hydrophilic chains. After that, micelles connect together with the bridge from other micelles. The sol-gel transition temperature can range from 21 to 40 °C, depending on the ratio of PEG to other hydrophobic polymer [68, 110]. However, there is no report on printing PECE or PLGA-PEG-PLGA yet. The PEG block polymers that can be printed by using extrusion based bioprinter include poly(*N*-(2-hydroxypropyl) methacrylamide lactate) with polyethylene glycol,

p(HPMAm-lactate)-PEG block polymer [9, 27, 55]. The bioprinted PEG block polymer has shown its potential for cartilage TE application [27].

Figure 2.7. PLGA-PEG-PLGA micelles gelation and hydrophobic interactions due to change in temperature.

2.1.2.3. Poly (N-isopropylacrylamide), PNIPAAm and derivatives

Polyacrylamide derivatives are another group of thermo-responsive hydrogels that are frequently used in drug delivery and biomedical applications [80, 111, 112], for examples, poly(N,N-dimethylacrylamide), poly(N-(2-hydroxypropyl)-methacrylamide lactate) and poly (N-isopropylacrylamide), PNIPAAm (chemical structure is shown in Figure 2.4) [80, 113]. PNIPAAm is the most popular one and gelled by both hydrophobic interaction and coil-globular gelation mechanisms at the LCST (approximately 30 – 37 °C) [55, 68, 70, 114]. Due to sol-gel transition at the human body temperature, PNIPAAm is an attractive material for bioprinting. Indeed, it has been printed by using pneumatic extrusion based bioprinter with Hyaluronan (HA) and other cell assembly techniques [34, 115]. Bioprinted PNIPAAm scaffolds could also be potentially used for cartilage TE [34]. However, like other synthetic polymers, it has low biocompatibility compared to natural based hydrogels and is not biodegradable [55, 102]. Therefore, PNIPAAm is usually used with other

polymers such as hyaluronic acid and alginate to improve its biocompatibility [56, 78, 112].

In conclusion, thermo-responsive hydrogels show their versatility and adaptability of changing sol-gel phase by change of temperature, and they are good candidate to be used as bioinks. They are grouped in two different categories based on origin of the materials which are naturally derived and synthetic. All the properties included gelation mechanisms, gelation temperature, concentration for bioprinting, post-print structure, cell viability and applications of mentioned hydrogel are summarized in Table 2.1.

Table 2.1. Thermo-responsive hydrogels: properties and gelation mechanism.

Thermo-responsive hydrogels	Gelation mechanisms	Concentration used in bioprinting	Gelation temperature	Bioprinting technique	Directly printed form	Material role in bio-printing	Cell viability		Applications	Ref.
							Cell types	% viability		
Natural derived thermo-responsive hydrogels										
Gelatin and derivatives	Coil-helix gelation	2 – 20 % w/v	< 35 °C	Extrusion, piezoelectric inkjet and 2PP	Grid pattern or tube in 2D-2.5D ^a	M & S	10T1/2 fibroblasts HepG2 hepatoma HUVECs ^b	> 80% (day 7) > 97% (day 7) > 75% (day 3)	Vascularization, cartilage TE, cell patterning and sacrificial materials	[55, 62, 64, 65, 68-70, 77-79, 116, 117]
Methylcellulose	Hydrophobic interactions	4 % w/v	~ 40 – 50 °C	Pneumatic extrusion	Grid pattern in 2D-2.5D	M	N.A.	N.A.	Rapid gelation agents	[68, 80, 81, 83, 84]

Agarose	Coil-helix gelation	1 – 5 % w/v	30 – 40 °C	Pneumatic and mechanic extrusion	Grid pattern or tube in 2D-2.5D ^c	M & S	Bone marrow stromal cells (BMSCs)	> 70% (day 7)	Sacrificial material and bone TE	[33, 55, 69, 83, 87, 88]
Collagen	pH crosslinking (neutralization) with thermal gelation	0.1 – 3% w/v	~ 37 °C	Extrusion and inkjet	Simple 2.5D shape	M	Keratinocytes Fibrochondrocytes	> 94% (day 1) > 90% (day 5)	Wound healing and Cartilage TE	[36, 55, 90, 92-95, 97]
Synthetic thermo-responsive hydrogels										
Pluronic and derivatives	Micelle interactions	~25 – 40 % w/v	10 – 40 °C	Pneumatic valve system, pneumatic and mechanic extrusion	Standing tube, grid, complex guided track and 3D mould	M & S	Bone marrow stromal cells (BMSCs)	< 5% (day 7)	Sacrificial material and soft tissue TE	[62, 70, 83, 98, 100, 101, 103, 104]

PEG based block polymer	Micelle and hydrophobic interactions	25 - 35 % w/v	21 – 40 °C	Pneumatic extrusion	Grid pattern in 2D-2.5D	M	Chondrocytes	~ 85% (day 3)	Cartilage TE and cell assembly	[9, 27, 55, 68, 108, 110]
PNIPAAM and derivatives	Hydrophobic interactions and coil-globular gelation	10 – 20 % w/v ^d	30 – 37 °C	Pneumatic extrusion	Grid pattern in 2D-2.5D	M	Chondrocytes	~ 98% (day 7)	Cartilage TE and bone regeneration	[34, 55, 56, 70, 78, 112, 115]

M = Model material, S = Support material or sacrificial material, Ref. = References

- a. Printed at 24-30 °C, b. Human umbilical vein endothelial cells, c. Printed at 40 °C and d. Total concentration of the hydrogels mixture not only for the PNIPAAM part

2.2. Bioprinting techniques classification

Figure 2.8. Sketch of three different types of bioprinting process.

Nowadays, there are many types of bioprinting techniques available for different applications. Depending on how the materials are delivered onto the platform, bioprinting systems can be categorised into three main types which are laser based, jetting based and extrusion based (dispensing), as shown in Figure 2.8 [18, 26, 31]. However, recent techniques such as magnetic bioprinting and electrohydrodynamic jetting have been used in tissue engineering as well. Table 2.2 summarises different types of bioprinting systems.

2.2.1. Laser based bioprinting

Laser based bioprinting can be categorised into two types: laser-guided direct cell printing and laser-induced cell printing. In laser guided direct cell printing, laser transfers cells from the original source (cells suspension) by pooling the cells using laser pulse. The difference in refractive indices between cells and cell media allows a laser to select and guide cells onto the substrate. In laser-induced cell printing as shown in Figure 2.8, the laser power is used to heat an absorbing donor layer (ribbon) to generate a change in pressure, which then leads to the ejection of a cell laden

hydrogel block or droplet below absorbing layer onto the selected substrate [18, 118, 119]. Both laser-based bioprinting techniques can provide high-resolution patterning (as shown in Figure 2.9a) [120], avoid clogging problem, and enable fabricating multi-materials (or multiple cell types) in a single structure with good cell viability. Nonetheless, they have some limitations, for examples, the hydrogels should be UV or chemical crosslinkable unless a special technique (e.g. spin coating) is applied [121, 122]. The material cost is higher and the processing time is usually longer than other techniques. Additionally, there is limited scalability [26, 35, 118, 119].

Recently, Ovsianikov *et al.* have used two-photon polymerization (2PP) technique to fabricate and pattern cell-laden photo-thermal sensitive hydrogels (combination of gelatin methacrylate (GelMA) and MG63 cells) as shown in Figure 2.9b [117]. Two-photon polymerization, also known as direct laser writing, is the 3D printing technology that uses a high intensity femtosecond laser. The focal point of the femtosecond laser is the source of two photons generation, which is able to create a localised chemical reaction (polymerization) inside a photopolymer resin (not on the surface). 2PP shows good resolution and avoids nozzle clogging problem [123]. Therefore, by using this technology with correct UV thermos-responsive polymers, complex structure can be fabricated. Stereolithography (SLA) is UV laser based 3D printing technique to cure photopolymer point by point on the surface of the resin and fabricate a 3D construct layer by layer. The building process of SLA can be top down or bottom up, depending on the position of the laser source, which make it possible to fabricate overhang structures without using support [124]. SLA has been modified to print a combination of polyethylene glycol dimethacrylate (PEDGA) and

GelMA hydrogels, in which the structure were fabricated at a high resolution with 85% cell viability [75].

Figure 2.9. **a)** Optical image of micro-array from LIFT technique (Reproduced with permission, Copyright 2007, Elsevier) [120], and **b)** 2PP bioprinted structure. Scale bar is 200 μm (Reproduced with permission, Copyright 2013, American Chemical Society) [117].

2.2.2. Jetting based bioprinting

Nowadays, many jetting systems have been explored as a bioprinter. The first jetting based bioprinting is inkjet technology. Inkjet is a well-established technology in 3D printing for scaffold and biomedical devices fabrication [125, 126]. Inkjet based bioprinting system can be further classified into two types: piezoelectric and thermal. The piezoelectric inkjet bioprinter ejects a droplet because the shape distortion of piezoelectric transducer creates an air bubble that expands to push the liquid out of nozzle. While thermal inkjet bioprinter uses heater to heat up the heat resistor and create bubble expansion to eject the droplet [17, 118, 127]. Usually, inkjet based bioprinter is used to print cell-laden droplets (as shown in Figure 2.8) at a high resolution in micro-scale (10–75 μm as structure shown in Figure 2.10a) [26, 35, 128]. Inkjet based bioprinter is not only used for *in vitro* studies but has also been proposed for *in vivo* bioprinting [129]. The advantages of inkjet bioprinter are low

cost, fast fabrication and high resolution [35, 118]. Hoch *et al.* showed that GelMA and modified methacrylated-acetylated gelatin can be printed using piezoelectric inkjet printer, but the viscosity of hydrogels need to be controlled, because at a higher viscosity the clogging problem is prone to occur [116]. In fact, one major drawback of inkjet printing systems is nozzle clogging. Moreover, other disadvantages include low cell density, drying of droplets during printing and limited 3D construction capability, unless using fast curing low viscosity hydrogels [18, 24, 26, 130]. Recently, a couple of new jetting based bioprinters have been developed such as valve based jetting printer and acoustic based jetting printer. Unlike conventional inkjet bioprinting, valve based printers control the ejection rate by opening and closing the valve, thus the size of droplets and number of cells are tuneable (the micro-valve bioprinted structure has been shown in Figure 2.10b) [127, 131, 132]. Moreover, valve based printers have two modes of printing: discrete droplet jetting and continuous stream jetting [104]. The advantages of valve based bioprinters include controllable droplet size, number of cells loaded, and a gentle releasing system which could lead to higher cell viability for fragile cell [131, 133]. However, valve based bioprinter can only print hydrogels that have a viscosity less than 200 mPa.s [134], it is more suitable for printing chemical crosslinkable hydrogels. In acoustic based bioprinter, the material is ejected out from the pool by acoustic wave. This technique does not rely on nozzle thus avoiding clogging problem (the schematic and printed structure have been shown in Figure 2.10c) [135]. However, heat is generated from the printing process [127, 131, 135], making it unsuitable to

print with thermo-responsive hydrogel since the heat may affect the hydrogel properties.

Figure 2.10. a) Florescent image of multi-cells inkjet bioprinted construct (Reproduced with permission, Copyright 2013, Elsevier) [128], b) Collagen type I and agarose hydrogel structure fabricated from micro-valve bioprinter (Reproduced with permission, Copyright 2016, John Wiley and Sons) [132] and c) Concept of acoustic bioprinter and structure (Reproduced with permission, Copyright 2007, Royal Society of Chemistry) [135].

2.2.3. Extrusion based bioprinting

Fused Deposition Modelling (FDM) technique is a popular extrusion based 3D printing technique that has been used to fabricate solid tissue engineering scaffold [136]. Extrusion based bioprinting system is similar in process but depositing cells and hydrogels. It is considered one of the most common and versatile technique. It can print hydrogels from low to high viscosity (from 30 mPa.s to 6×10^7 mPa.s). Therefore, extrusion based bioprinter can deal with a broad range of hydrogels from photo-crosslinkable hydrogels to thermo-responsive hydrogels [119, 137]. In fact, most of thermo-responsive hydrogels (e.g. gelatin, pluronic and methylcellulose) have been fabricated by using extrusion based bioprinter [35, 65, 83, 138]. Extrusion based bioprinter is driven by pneumatic force (air pressure) or mechanical force (using screw or piston) to extrude cell laden hydrogel out of the nozzle as shown in Figure 2.8 and bioprinted structure has been shown in Figure 2.11a [26, 64, 119]. The advantages of this techniques are good scalability, high cell density and the ability to create 3D vertical structures [24, 64, 75, 118, 119].

Although this technique is quite versatile and flexible, the resolution of this technique is low (a few hundred micrometre to millimetre) compared to other techniques. Furthermore, when hydrogel is more viscous, higher force (shear stress) is required for extrusion. As a result, the cell viability in viscous hydrogel from extrusion bioprinter might be lower than other techniques due to the applied shear stress effect on cell suspension. [35, 55, 119]. Recently a hybrid technique has been developed to improve cell viability. The hybrid technique involves at least two types of material, one acts as support structure to provide mechanical strength, and the other cell-laden hydrogel is used for printing inside the support structure (Figure 2.11b). By using this hybrid system, the processing speed as well as cell viability is increased [139, 140].

Figure 2.11. **a)** Direct extrusion bioprinted scaffold (Reproduced with permission, Copyright 2013, Elsevier) [64] and **b)** Schematic of hybrid extrusion bioprinting system and structure. Scale bar: 400 μm (Reproduced with permission, Copyright 2014, Nature Publishing Group) [140].

2.2.4. Recent developments in bioprinting technologies

Magnetic bioprinting is a new technique that incorporates magnetic nano-particles with cells to create magnetic tissue spheroid [141, 142] for use in a magnetic levitation system. The magnetic levitation system or magnetic bioprinting forms and

drives the formation of tissue spheroids under the magnetic field without using scaffold or support material. Moreover, this system shows that the cell viability is high when compared to other systems because of less impact load to cells [141-143]. However, long term effects of nanoparticles on cell differentiation and human toxicity are still elusive, and this system needs more pre-clinical tests to prove its safety [144].

Electrohydrodynamic jetting is another emerging technology that can print at less than 100 nm. Unlike inkjet printing, the mechanism of electrohydrodynamic jetting starts with the input of a high voltage between nozzle and platform (similar to electrospinning) to enable bioinks to jet [145]. Electrohydrodynamic printing has been applied to electronic applications due to its high resolution for patterning [145, 146]. In tissue engineering, it has also been used for hydrogel patterning to study cell interactions to substrate at different surface topography conditions. Moreover, a report has shown that cells can survive and be directly printed in the electrohydrodynamic system by using a larger nozzle. However, this technique is unable to create sizable 3D structures. The total height or thickness of the printed part is relatively low compared to other 3D bioprinting techniques [127, 145].

Table 2.2. Summary of current bioprinting techniques.

Bio printing technology	Advantages	Disadvantages	References
<i>Laser based</i>			
Laser-guided direct cell printing	High resolution High cell viability No clogging	High printer cost Slow fabrication process Limited scalability	[18, 119]
Laser-induced cell printing	High resolution High cell viability Multi-materials fabrication No clogging	High printer cost Limited scalability Metallic residuals contamination	[26, 118, 119, 121, 122]
Stereolithography (SLA) and two photon polymerization (2PP)	High resolution High shape fidelity in vertical 3D structure No clogging	Laser effect on cell viability Hydrogel needs to be UV-photo crosslinked	[75, 117, 123, 124]
<i>Jetting based</i>			
Piezo inkjet printing	High resolution Availability of machine Fast	Clogging Low cell density Low shape fidelity in 3D	[17, 24, 26, 127, 130]

		Low shape complexity in 3D Limit bioink viscosity Drying of droplets	
Thermal inkjet printing	High resolution Fast Low cost	Clogging Low cell density Low shape fidelity in 3D Heat may affect hydrogel properties Non-uniform droplets Drying of droplets	[17, 26, 118, 130]
Valve based printing	Medium to high resolution Good cell viability Less clogging Able to jet from droplet to strand	Low shape fidelity in 3D Time delay	[17, 35, 104, 127, 131]
Acoustic printing	High resolution Uniform droplets size Less clogging	Low shape fidelity in 3D Low cell density Limited bioink viscosity	[17, 118, 127, 131, 135]

<i>Extrusion based</i>			
Pneumatic extrusion printing	Versatile with wide ranges of bioink High shape fidelity in 3D structure High cell density	Low resolution Shear stress effect on cell viability Time delay	[26, 35, 83, 118, 119]
Mechanic extrusion printing	Versatile with wide ranges of bioink High shape fidelity in 3D structure High cell density Good scalability Direct control to material flow	Low resolution Shear stress effect on cell viability	[26, 35, 65, 118, 119]
<i>Recent developed techniques</i>			
Magnetic printing	High resolution Support material free High cell density	Toxicity of nano-materials High system cost	[141-144]
Electrohydrodynamic jetting	High resolution Good precision control Good cell viability	High voltage and special setup Low shape fidelity in 3D form	[127, 145, 146]

2.3. Roles of support materials in bioprinting

Recently, printing beyond 2.5D (2.5D shape is the shape that comes from repeatedly printing a 2D pattern in Z direction without changing the pattern in any layer) grid structure and simple tubes into the micro-channeled and hollow structures represent a great advancement in bioprinting [33, 62]. It is no longer stacking layer by layer in the same pattern. To form small channels or hollow tubes, a second “bioink” is needed. The second bioink serves as support material or sacrificial material. This technique normally comprises of chemically crosslinked hydrogel especially UV crosslinked hydrogel such as GelMA [62] or polyethylene glycol diacrylate (PEGDA) [147] as model materials and usually thermo-responsive hydrogels such as pluronic F127 or agarose as support materials [148]. By incorporating both types of hydrogels, a complex hollow or complex track pattern in 2.5D level or microfluidic level can be fabricated.

Even though the complex track has more advancement compared to just 2.5D simple grid or lattice structure, in the reality, the real human organ is not in 2.5D plane. Rather, human organ is an intricate 3D structure which has a very complex shape, fine details and topography. Therefore, the technique in Figure 2.6d is not adequate to bring 3D bioprinting to the level of printing a human organ. This is due to the fact that only one of the materials, either model or support materials (mostly support material), has good mechanical strength which is responsible for the shape stability and shape integrity.

Another recent advancement is the development of freeform reversible embedding of suspended hydrogels or “FRESH” technique, which allows 3D structure to be fabricated by using gelatin bath as the support material with many types of other hydrogels as model materials such as alginate with calcium

chloride (CaCl_2) or fibrinogen and thrombin [79]. However, this technique needs precision control in the temperature in addition to positional control. Gelatin is thermo-responsive which will start to melt when the temperature is above 30 °C [148]. Therefore, temperature control is critical for “FRESH”. Moreover, the “FRESH” technique is batch by batch specific so the crosslinked agent must be pre-mixed with gelatin bath first. Lastly, because of gelatin bath, the printing temperature needs to be lower than 30 °C (around 22-25 °C) which is not ideal for printing cells over a prolonged period. To date, “FRESH” is one of the most advanced techniques. To sum up, the list of support materials for bioprinting is shown in Table 2.3.

Table 2.3. Common support hydrogels in bioprinting.

Support hydrogel	Model hydrogel	Bioprinted form	References
Gelatin and derivatives	Alginate, Fibrin and collagen	3D hollow structure	[79]
Agarose	GelMA, SPELA*, PEGDMA** and PEGDA	2.5D complex track	[88]
Pluronic and derivatives	GelMA and Agarose	2.5D complex track and micro-pattern	[62, 104]

*Star poly(ethylene glycol-co-lactide) acrylate

**Poly(ethylene glycol) dimethacrylate

In 3D printing, support materials always play an important role to create overhanging and hollow structures. As shown in Figure 2.12 which is an anatomical heart model, the overhanging part (Figure 2.12a) needs a support to hold as in other 3D printing techniques (Figure 2.12b). It is almost impossible for bioprinting to fabricate this structure without using support. However, comparing

the materials that have been used in Fused Deposition Modelling (FDM) with hydrogels or bioinks for bioprinter, the mechanical strength of the FDM materials is typically stronger [149, 150]. The current hydrogels that have been used with bioprinter are too soft to hold the shape. Moreover, with the high water content, the osmosis pressure will affect the interaction between model and support materials (e.g. water travels by osmosis pressure to another hydrogel which will lead to change in concentration, viscosity and mechanical properties). Thus, the requirements of bioprinting for fabricating 3D complex hollow structures are: (i) the material (including both model and support) must have sufficient shape integrity and can be printed in a cell friendly environment. To ensure biocompatibility properties, naturally-derived materials or even extracellular matrix (ECM) derived materials are preferred. Furthermore, to enhance mechanical properties, strong naturally-derived materials such as silk or hydroxyapatite can be integrated with the model hydrogel as the composite natural materials; (ii) there is no or minimal reaction between model and support materials; and (iii) support materials must be easily removable without sacrificing cell viability. In order to achieve this property, either the concentration of model and support materials need to be similar or there is an interaction to create wall between model and support materials. For example, if model material contains Ca^{2+} and support contains alginate, the wall can be created by semi physical crosslinked reaction of alginate and calcium ion. However, all the mentioned techniques need the advancement of materials science and chemistry to better understand the nature of materials and reaction mechanism.

Figure 2.12. a) Computer model of the anatomical heart (red arrows pointed the overhanging parts) and b) Computer model of the anatomical heart with support structure.

2.4. Summary of thermos-responsive hydrogel for 3D bioprinting

Bioprinting is a new and emerging field with immense potentials. It addresses interface issues between robotic automation and living materials. Bioprinting has good repeatability, high precision and adequate scalability, which are important for tissue substitute fabrication and may potentially lead to organ printing. Thermo-responsive hydrogel plays important roles in bioprinting. It is a good candidate for bioprinting because it has a unique characteristic of reversible sol-gel transition upon temperature change and an adjustable printability by controlling physical crosslinking. Thermo-responsive hydrogels are very useful for TE applications and have become part of the advancement in bioprinting technology.

Nevertheless, there are some challenges to be resolved. For example, vascularization remains as the main problem in order to provide long term sustainability for developing tissue to organ [151]. We are in need of highly printable hydrogels (as shown in Figures 2.6 and 2.11) that can help create vascularization, while possessing bioactive features to provide a suitable environment for cells to grow. The current issue of hydrogel is that some are good in biocompatibility but poor in printability (either low shape fidelity or low

resolution) or vice versa. Thus, it is very difficult to create a 3D complex structure. Lastly, just good printability and bioactivity may not be sufficient for TE, because hydrogels for TE applications must also provide adequate mechanical strength to support tissue construct. Novel thermo-responsive hydrogels must be developed in order to meet all of these TE requirements. The development of novel bioinks will be further described in Chapter 4.

2.5. Biodegradable film and membrane fabrication

There are two main principles in the fabrication of the film and membrane, namely, polymer solution (Figure 2.13a) and polymer melt (Figure 2.13b). The polymer solution principle applies to processes that require the use of organic or inorganic solvents. The polymer chains in the solvent will entangle and intersect further with the increase in concentration as the solvent evaporates.

Figure 2.13. Schematic of transformation of polymer chains: **a)** polymer chain in solution from low to high concentration and **b)** polymer chain in polymer melt form from low to high temperature.

When solvent evaporation is completed, the remaining polymer in the form of solid can be obtained [152]. Membrane fabrication techniques developed based

on this principle include solvent casting technique with or without porogens, freeze drying, spin casting and electrospinning.

In contrast, polymer melt principle is applicable to processes that require the use of heat to make polymer flow, thus allowing the polymer to combine into a homogeneous piece. Figure 2.13b shows an example of semi-crystalline polymer. At a high temperature (above the melting point of polymer), the polymer chains flow and change into amorphous state [153]. When the polymer melt cools down, it turns back into solid form and the chains will re-align to form the crystal structure. Membrane fabrication techniques developed based on this principle are tooling techniques such as two-roll milling, electrospinning and melt spinning. Film and membrane fabrication methods described in this section are organic solvent (solution) casting, water based casting technique, particulate leaching, freeze drying (extraction), gas foaming, spin casing, two-roll milling and electrospinning. They are grouped in three different categories as shown in Figure 2.14 The processing methods are categorized systematically into basic casting technique, porogen technique and tooling technique. Thickness (in film or membrane form), process duration, advantages and disadvantages of each method are summarized in Table 2.4.

Figure 2.14. Classification of film and membrane fabrication processes

2.5.1. Basic casting techniques

In basic casting techniques, no porogens is used and there is minimum equipment set up. A polymer-solvent mixture is left for several hours to days in order to allow solvent evaporation. In this category, two methods, namely solvent casting and water based techniques, are presented.

2.5.1.1. Solvent casting

Solvent casting or solution casting is one of the most conventional methods to make biodegradable film. As illustrated in Figure 2.15a, solvent casting method begins by mixing biodegradable polymer such as polycaprolactone, (PCL) and poly(l-lactic) acid, (PLLA) [1] or composite between natural and synthetic materials like oxide starch/chitosan/graphene oxide [154] with organic solvent.

Subsequently, the mixture is left for 6 hours to 2 days in a fume hood until the organic solvent is fully evaporated. Thereafter, the remaining solid film can be obtained by peeling it off the container manually. Vaquette *et al.* [1] had reported that pore structure could be found on the film surface. However, most of solvent casted films are solid films without any pore. Surface post-processing is required in order to create or increase the number of pores in the film. The thickness of film from this method can be varied based on the polymer types and solution concentrations which range from 35 to around 180 μm [155-157].

2.5.1.2. Water based casting technique

Water based technique is similar to solvent casting. Instead of using organic chemicals, the main solvent for this method is water. This process is suitable for making hydrophilic natural polymer films or membranes such as chitin hydrogels [158] or even polymer composites e.g. fibrillated cellulose, polyvinyl alcohol, (PVA) [159] and chitosan with polyethylene glycol (PEG) [160]. For a biodegradable film that contains chitosan, in addition to water as the main solvent, a small amount of acid is also required to increase the solubility of chitosan [161]. The fabrication process starts with the mixing of polymer with water or acidic solution. Next, the mixture is stirred continuously until a homogeneous solution is achieved. The overall mixing process takes around 1 to 3 hours [159, 160]. However, if there are other processes required such as UV irradiation for crosslink gel [161] or drying process for dehydrated film [160], the overall process duration may need an additional 30 minutes to 24 hours. In comparison to solvent casted films or membranes with elastic modulus up to 550 MPa [156], natural films made from this technique have only an approximate maximum elastic modulus of 20 MPa [160]. This leads to problems such as poor mechanical

strength and fast degradation rate [36]. Hence, these films and membranes cannot be used as standalone scaffolds without prior strengthening with synthetic polymers or other strengthening agents such as graphene oxide [154]. The thicknesses of water based films and membranes range from 25 to 200 μm . These thicknesses of films or membranes are based on solution concentrations, the amount of water content and swelling properties of material [159, 160, 162, 163].

2.5.2. Porogen techniques

The main objective of this technique is to create or increase porosity of the membrane in a single step. Porogens can be in the form of solid particles, ice crystals or gas bubbles which are leached out by the principle of phase separation. Three techniques will be discussed.

2.5.2.1. Particulate leaching

Particulate leaching method is similar to solvent casting except that it involves porogens for rapid creation of pore structure during casting. This method is able to create a highly porous structure [164]. The porogens can be salt, wax, sugars or even hydrophilic polymer such as PEG [5, 165, 166]. The schematic diagram of this method is shown in Figure 2.15b. Starting off like solvent casting, polymer mixture is first being prepared. Secondly, the polymer solution is poured into a porogen mould after mixing. Third, the solvent is left for 8 hours up to a few days for full evaporation [167]. Last, a solvent, usually water, is used to leach out the porogens. This technique is able to control pore size but not pore configuration. In this case, the pattern of pores is random [5, 164]. Porosity more than 93% and pore diameters up to 500 μm on average can be achieved using this method. The

membrane thickness from this method ranges from 20 to 200 μm depending on polymer concentration [166] and solidification time [167].

Figure 2.15. Schematic of **a)** solvent (solution) casting film fabrication, **b)** particulate leaching membrane fabrication **c)** SEM image of solvent casted film and [154] **d)** SEM image of porogen leaching membrane [166].

2.5.2.2. Freeze drying

The freeze-drying method illustrated in Figure 2.16a involves the mixing of polymer powders or pellets with an organic solvent to form polymer solution. This solution is frozen at a very low temperature. Under high vacuum condition, the solvent is removed through phase separation processes which include sublimation, extraction or evaporation between polymer with rich phase and poor phase. Finally, a membrane with high porosity and interconnected micro-pores is formed. Freezing processing time ranges from seconds (quenching) to 20 minutes.

[5, 164, 168, 169]. Porosity of freeze drying membrane can be altered by varying the concentration of the solution and temperature during the freezing process. One example of freeze extraction work is the PLLA/PCL membrane produced by Gaona *et al.* In this work, PLLA/PCL was dissolved in dioxane and later froze in liquid nitrogen. Pores appeared by washing away the dioxane using ethanol [169]. Thickness of freeze drying membrane can be controlled within the range of 20 to 200 μm by adjusting the amount of polymer and freezing rate [170].

Figure 2.16. Schematic of **a)** freezing extraction fabrication method, **b)** gas foaming fabrication method, **c)** SEM image of cross sectional of the freeze extraction membrane [169] and **d)** SEM image of cross sectional of gas foaming membrane [171].

2.5.2.3. Gas foaming

In this method, supercritical fluid (SCF) is used as an immiscible component. Supercritical fluid refers to any substances present at a temperature and pressure

condition higher than its critical point [172]. The common fluid used in this method is carbon dioxide or CO₂ [157, 171]. The gas foaming method is described in Figure 2.16b where polymer is combined with CO₂ gas and CO₂ starts to dissolve at a lower glass transition temperature (T_g) and high pressure condition. The process begins with pre-conditioning at a specific pressure for a period of 1 hour to 17.5 hours (for saturation condition), followed by desorption and foaming process which takes around 10 to 20 minutes [157, 173]. The number of pores and pore interconnectivity depends on the amount of CO₂ used [5, 172]. Other parameters such as depressurizing rate and cooling rate also affect foaming properties [174]. Although this method can generate a highly porous structure, the limitation is that it is only suitable for polymers that have low T_g [172]. The thickness of membranes made from this method (which based on initial film thickness) is usually around 150 to 180 μm [157, 175].

2.5.3. Tooling techniques

Tooling techniques require the use of a special tool to fabricate films or membranes. Some examples of such tools are spinning machine, roll-milling machine, electrospinning and fibre related techniques as described below.

2.5.3.1 Spin casting

Spin casting or spin coating involves polymer mixture preparation which is similar to many fabrication methods. However, as illustrated in Figure 2.17a, instead of static casting, this method can create smooth films on a spinning plate. A solid film will be obtained after solvent evaporation step [176]. Total process duration depends on mixing time, spinning time and solvent evaporation time which can take less than 1 hour or to a maximum of 3.5 hours [177]. The advantage of this method is that it can fabricate a film with evenly distributed

thickness. Patterned porous membrane made by post-processing with polymeric moulds or etching of sacrificial material can also be generated through this method [177]. There is a similar work from Kasuya *et al.* (2012) which combined the principle of phase separation and spinning coating to cast PLGA in water-dioxane solution system. This study reported that a membrane could be fabricated directly without surface modification and pore size and porosity can be varied by moisture [178]. Usually, films and membranes made from this method are very thin and the thickness ranges from 2 to 6 μm [176, 178]. However, combination of spin coating and drop-coating method can control the thickness of membrane from 0.5 to 80 μm [177].

2.5.3.2. Two-roll milling

Figure 2.17b shows the schematic of two-roll milling film fabrication with polymer pellet or powder. Solid polymer is melted and milled into solid masses are formed by the two rotating cylinders. The speed and temperature for this method are dependent on polymer properties. The use of solvent is eliminated in the fabrication of films using this method. As a result, post-processing is necessary to address the inadequacy in porosity and unevenness in thickness [176].

In order to create a thinner film, solid masses need to undergo hot pressing with stainless steel plates for around 10 minutes. The thickness of film after pressing process is around 95 to 170 μm [176, 179].

Figure 2.17. Schematic of **a)** spin casting, **b)** two-roll milling film fabrication method and **c)** SEM image of spin casting film [177].

2.5.3.3. Electrospinning and fibre related methods

The electrospinning method gathers the most interest as it can produce fibres in nano scale and form membrane structures that mimic Extra Cellular Matrix (ECM) of native tissue [180]. Nowadays, there are many TE applications of electrospun membrane such as blood vessels [47], cartilage [181] and bone [182, 183]. Most electrospinning methods start with polymer solution similar to solvent casting method as shown in Figure 2.18. However, there is a report showing that polymer liquid (polymer melts) can be prepared by the use of heat [184]. Instead of merely casting with the solution casting method, solution is filled in a syringe and extruded out in fibre form using high voltage electricity. Thereafter, fibres stack randomly onto the platform to form a membrane within a few minutes to an hour depending on the length of fibres and layer thickness [185, 186]. The size

of electrospun fibre is in the range of micro to nanometre size based on the properties of solution, operating condition and environment conditions. The pore size of electrospun membrane is subjected to the size of fibre where smaller fibre will lead to smaller pore size [187]. Thickness of electrospun membrane ranges from 30 to 200 μm and this relies on solution concentration and extrusion rate [188, 189]. Nevertheless, there is a report stated that by using gelatin coating technique, composite materials electrospun membrane can reach a thickness less than 3 μm [190]. The main challenge of electrospun membrane is to develop a precise way for the stacking of fibres [191] such that the dense morphology structure which prevents the migration of cells through interior structure can be avoided [192]. Wright *et al.* has devised a possible solution for dense structural problems in electrospinning. By adopting similar principle as particulate leaching, sodium chloride (NaCl) salt crystals were used to improve porosity of electrospun membrane [193].

Apart from electrospinning techniques, there are other existing technologies that have the potential to fabricate membranes through micro-nano fibres. Melt spinning is one such technique that can create aligned fibres by using polymer melt principle and eliminate the use of organic solvent. Low production rate is one of the disadvantages of electrospinning. However, as compared to other methods, this method is able to generate a larger quantity of products [194, 195]. Currently, there are pressurized gyration and centrifugal jet spinning techniques that possess the ability to increase the production rate to 6 kg/h or more. These two techniques require a rotation motor to spin out the fibre in a circular manner towards the collector [196, 197]. The difference between these two processes is that pressurized gyration uses pressurized gas to jet out the polymer solution from

the orifice [196] whereas centrifugal jet spinning uses a syringe pump to jet polymer solution into the system [197]. These two techniques bring about improvement in the production quantities of micro-nano fibres to serve the future demand in TE applications.

Figure 2.18. a) Schematic of electrospun membrane fabrication method and **b)** SEM image of electrospun membrane [191].

Table 2.4. Comparison of film and membrane fabrication methods

Method	Thickness	Process Duration	Advantages	Disadvantages	References
Solvent casting (Solution casting)	35 – 180 μm	Several hours to a few days	<ul style="list-style-type: none"> • Versatile; can be used with most of biodegradable polymer • Simple: No special equipment set up 	<ul style="list-style-type: none"> • Involve organic solvent; require purifying process • Insufficient number of pores 	[1, 154-157]
Water based Techniques	25 – 200 μm	A few hours to a day	<ul style="list-style-type: none"> • Suitable for natural and hydrophilic biodegradable polymer • Little or no organic solvent involved 	<ul style="list-style-type: none"> • Not applicable to use with hydrophobic polymer • Relatively low mechanical strength 	[158-160, 162]
Particulate leaching	20 – 200 μm	Several hours to a few days	<ul style="list-style-type: none"> • Versatile; can be used with most of biodegradable polymer • Able to create a high porous structure directly • Pore size can be controlled 	<ul style="list-style-type: none"> • Involve organic solvent; require purifying process • Random pore configuration 	[5, 165-167]
Freeze drying (Freeze extraction)	20 – 200 μm	A few to several minutes	<ul style="list-style-type: none"> • Able to create highly porous structure directly • Porosity can be varied based on solution concentration and freezing temperature 	<ul style="list-style-type: none"> • Require special equipment set (freezer and vacuum pump) • Random pore configuration 	[164, 168-170]
Gas foaming	150 – 180 μm	Several hours (Within a day)	<ul style="list-style-type: none"> • Able to create highly porous structure directly • No organic solvent involved 	<ul style="list-style-type: none"> • Require special equipment set up for SCCO₂ • Suitable only with polymers that have low T_g 	[157, 171-175]

			<ul style="list-style-type: none"> • Porosity can be varied based on amount SCCO_2, depressurized rate and cooling rate 		
Spin casting (Spin coating)	0.5 – 80 μm	A few hours	<ul style="list-style-type: none"> • Versatile; can be used with most of biodegradable polymer • Even thickness 	<ul style="list-style-type: none"> • Involve with organic solvent; require purifying process • Insufficient pore structure except combining with porogens techniques 	[176-178]
Two-roll milling	95 – 170 μm	Several minutes	<ul style="list-style-type: none"> • Versatile; can be used with most of biodegradable polymer • No organic solvent involved 	<ul style="list-style-type: none"> • Require milling machine • No pore structure • Uneven film thickness 	[176, 179]
Electrospinning & fibre related methods	3 – 200 μm	A few minutes to an hour	<ul style="list-style-type: none"> • Versatile; can be used with most of biodegradable polymer • Able to create high porous structure directly • Able to mimic ECM structure • Able to create nano-scale structure • Some methods have high production rate 	<ul style="list-style-type: none"> • Involve with organic solvent; require purifying process except for melting technique • Too narrow pore size and dense structure requires surface modification • Require high voltage supplies or special equipment setup • Random pore configuration 	[184-186, 188-192, 196, 197]

2.6. Post-processing

Majority of the film fabrication methods require post-processing to create pores on the surface. Moreover, some TE applications require reduced film thickness and so post-processing to make a thinner film is necessary. For some of the biodegradable polymers such as PCL and PLLA which are hydrophobic [198, 199], surface modification is required to reduce the hydrophobicity for enhanced cell attachment. Additionally, some fabrication techniques involve organic solvents, thus the additional purifying process is required to remove any chemical residues. All the post-processing approaches are summarised in Table 2.5.

Table 2.5. Summary of film and membrane post-processing method

Post-processing	Modifying approaches	Modification outcome	Process duration	References
Physical surface modification	Laser trimming	<ul style="list-style-type: none"> • Larger pore size and higher porosity • Patterned pore structure 	3 – 10 minutes	[155, 189, 200]
	Robotic punctuation system	<ul style="list-style-type: none"> • Larger pore size and higher porosity • Patterned pore structure 	5-10 minutes	[201]
	Heat treatment	<ul style="list-style-type: none"> • Higher strength 	30 - 60 minutes	[159]
Chemical surface modification	Alkali (e.g. NaOH) treatment	<ul style="list-style-type: none"> • Hydrophilicity of surface increases 	A few hours to a few days	[66-68]
	Plasma treatment (e.g. O ₂ & Ar)	<ul style="list-style-type: none"> • Hydrophilicity of surface increases • Higher porosity • Preparation for grafting and immobilisation 	1 – 5 minutes	[165, 202, 203]
	Immobilize with hydrophilic polymer	<ul style="list-style-type: none"> • Hydrophilicity of surface increases 	Several hours to a few days	[204-206]

		<ul style="list-style-type: none"> • Better cell attachment 		
Purifying	Cleaning using water bath	<ul style="list-style-type: none"> • Film or membrane without organic solvent residues 	1 – 2 days	[207]
	Soaking and rinsing with ethanol	<ul style="list-style-type: none"> • Film or membrane without organic solvent and acid residues • Pure membrane without excess porogens (e.g ice crystal) left 	1-2 days	[168, 208]
	Using desiccator or vacuum drying	<ul style="list-style-type: none"> • Film or membrane without organic solvent and acid residues • Dried film and membrane (after wetting purification) 	1 -3 days	[169, 209]
	Rinsing with NaOH	<ul style="list-style-type: none"> • Film or membrane becomes neutral 	10 – 15 minutes	[160, 200]
	Rinsing with organic solution or hot water	<ul style="list-style-type: none"> • Pure film or membrane without excess uncross-linked polymer 	10 -15 minutes	[163, 210]

2.6.1. Surface modification

Surface modification can be separated into two groups based on their characteristics namely, physical and chemical. Physical approaches involve laser, punctuator and heat treatment. On the other hand, chemical approaches use chemicals that are in gas, liquid or gel form.

2.6.1.1. Physical modification

Porosity is an important characteristic of TE scaffold [211] and a high porosity on film surface is necessary. Fabrication methods such as solvent casting and two-roll milling cannot generate sufficient pores. Physical post-processing used for creating pores and patterned pore structure includes laser modification [155, 200] and robotic punctuation [201] as demonstrated in Figure 2.19. These methods can generate pores within a few minutes. For electrospun membrane, even though this method can create porous structure, the pore size is too small in a dense structure. Thus, laser modification is applied to electrospun membrane to improve pore size and resolve dense structure issue [189]. Lastly, for composite natural polymer, some post-processing is required to cure the polymer film, for example, heat treatment using oven or hot compression for around 30 minutes [159].

Figure 2.19. a) Optical microscope image of laser modified film [155] and b) SEM image of robotic perforated film [201].

2.6.1.2. Chemical modification

In addition to porosity, suitable surface chemistry is also significant for cell attachment and cell growth in the TE scaffold [5, 206]. Some of the biodegradable polymer films or membranes, for example, film or membrane that is made from PCL and its composite, need to undergo surface modification to reduce their hydrophobicity for better cell adhesion. Common surface

modification methods are alkali treatment for a duration ranging from several hours to a few days, depending on the solution concentration and sample size [212-214], as well as gas plasma treatment (e.g. oxygen, O₂ and argon, Ar) that requires only a few minutes [165, 202, 206].

Furthermore, Ar plasma can enhance the surface hydrophilicity and introduce peroxides and hydroperoxides functional groups onto the surface. Surface hydrophilicity and cell biocompatibility can be further improved with polymerized (UV grafting) or immobilized hydrophilic natural polymer (e.g. collagen and gelatin) after Ar plasma. The total process can last from 5 hours to a day [203-205]. Similarly, O₂ gas plasma can be used to decrease surface hydrophobicity as well as to increase the surface porosity [215].

2.6.2. Purifying

Though organic solvent, for example, chloroform, is suitable for dissolving hydrophobic polymer such as PLLA, PCL and their blends [1, 47, 216], traces of organic solvent in scaffolds are harmful to cell and tissue. In order to use film and membrane safely for TE scaffold application, it is necessary to remove any solvent residues by purifying them. Purifying method normally starts with rinsing film or membrane with deionized water several times [217]. Then, the sample is further purified by soaking in the water bath for 1 to 2 days [207]. For freeze extraction membrane, it requires the use of ethanol to get rid of the solvent and this process usually takes around 1 to 2 days [168, 208]. As acid is used during the fabrication process of film or membrane made from natural polymer such as chitosan and cellulose purification, NaOH is required to neutralize the acid residues for 10 to 15 minutes [160, 200]. For cross-linked fabrication process, left over polymers from the excessive incomplete reaction can be removed by

washing with organic solvent (such as acetone) or hot water for a few minutes [163, 210]. Lastly, film and membrane that involve solvent require a drying process in desiccator or vacuum oven for 1 to 3 days to get rid of solvent residues [169, 209].

2.7. Summary of biodegradable membrane fabrication and modification techniques

To sum up, biodegradable polymer film and membrane have shown their potential for TE applications. However, current fabrication processes are limited to mainly solvent-based approaches and lack control of pore size and pores structure. Some processes are subject to the availability of equipment and may not be readily available. Moreover, bioactivity and functionalization of the film/membrane usually requires one or more separate processes. There is almost no case in which a bioactive, functionalized and porous membrane is rapidly produced in a single process without using organic solvent. These challenges should be addressed in future study when developing new film/membrane fabrication processes for tissue engineering scaffold to serve as cell carrier. In addition, fabrication technique of film and membrane need further modification in order to enhance biological properties. Therefore, in this work, the membrane surface modification has also been explored. The details will be discussed in Chapter 6.

Chapter 3 Mathematical modelling of hydrogel printing resolution

Pneumatic extrusion-based bioprinting is a recent and interesting technology that is very useful for biomedical applications. However, many process parameters in the bioprinter need to be fully understood in order to print at an adequate resolution which is the most important factor that affects quality of the printed structure. In this chapter, a simple yet accurate mathematical model to predict the printed width of a continuous hydrogel line is proposed, in which the resolution is expressed as a function of nozzle size, pressure and printing speed. A thermo-responsive hydrogel, pluronic F127, is used to validate the model predictions. This mathematical model will provide a guide for subsequent studies on pneumatic extrusion-based bioprinting as well as for developing new bio-ink formulations which will be further investigated in Chapter 4.

3.1. Introduction

Bioprinting is an emerging technology that integrates robotic dispenser with multi-materials and living materials. It has brought many benefits to tissue engineering and other biomedical applications by fabricating customized tissue constructs and prostheses [23, 218, 219]. A typical bioprinting system consists of three components including: (i) living materials which mostly refer to cells; (ii) hydrogel or bio-ink; and (iii) a bioprinter [31, 220]. For bioprinters, there are mainly three types: laser-based bioprinters, ink-jet-based bioprinters and extrusion-based bioprinters [12, 60]. Among the three bioprinting systems, an extrusion-based bioprinter has the highest capability to deal with the widest range of biomaterials at a viscosity range from 30 to 6×10^7 mPa·s [18, 35, 118]. Hence, an extrusion-based bioprinter is considered one of the most versatile techniques and has been extensively used for biomedical applications [62, 104, 134, 136,

221]. Furthermore, recent research demonstrates that the electrospinning process can also be re-designed to have extrusion-like capability for the controlled printing of polymers and cells [15, 222].

Bioink or hydrogel serves as a support material for cells and is one of the most important components in an extrusion bioprinting system. Many synthetic and natural hydrogels have been used in bioprinting (as bioink) and biofabrication, such as polyethylene glycol (PEG) block polymer, hyaluronic acid, collagen and alginate [22, 31, 59]. However, few research works have been trying to understand and predict how the properties of bio-ink and the printing parameters relate to the final form of the printed construct. For example, Bruneaux *et al.* (2008) showed how viscosity relates to shear rate inside the nozzle and how applied pressure relates to the materials flow rate [223]. This work provides a basic understanding of a micro-extrusion bioprinting system. Cheng *et al.* (2008) focused on cell-laden hydrogel behaviour and cell viability on extrusion [224]. This interesting work showed the relationship between cell viability and shear stress, but not the relationship between viscosity and the resolution of the printed scaffold. Lee *et al.* (2015) developed a mathematical model on the relationship between printing speed and resolution; however, important parameters such as applied pressure and materials viscosity were not considered in this model [225]. Therefore, there is a need for further development on how materials' properties and printing parameters affect the printed width (resolution) of hydrogel constructs, as it reflects the shape fidelity and complexity that the system can achieve. In this work, pluronic F127 was selected as model material and a simple yet accurate model was developed to understand the influence of printing parameters on a hydrogel strand. In particular, how the printing resolution is

related to the size of the nozzle, stage moving speed and gauge pressure to print a continuous hydrogel strand is examined.

3.2. Mathematical modelling of line width, gauge pressure and stage moving speed

As shown in Figure 3.1, the pneumatic extrusion dispenser system is similar to capillary rheometer [223, 224, 226], which can be modelled by the following equations.

Figure 3.1. Schematic of pneumatic extrusion.

First, the maximum stress in capillary flow (τ_w) [226] can be found from

$$\tau_w = \frac{R\Delta P}{2L} = \frac{D\Delta P}{4L} \quad (1)$$

where R is the radius of the nozzle (m), D is the diameter or size of the nozzle (m), L is the length of the nozzle (m), and ΔP is the pressure drop through the reservoir and nozzle (Pa).

The shear rate in capillary of power law flow ($\dot{\gamma}_w$) [226] can be found from

$$\dot{\gamma}_w = \frac{3n + 1}{4n} \cdot \frac{4\dot{Q}}{\pi R^3} = \frac{3n + 1}{4n} \cdot \frac{32\dot{Q}}{\pi D^3} \quad (2)$$

where $\dot{Q}$ is the volumetric flow rate (m^3/s) and n is power index which when $n = 1$, it means that the fluid is Newtonian fluids whereas when $n \neq 1$, fluid is non-Newtonian fluids. For hydrogel, $n < 1$ which also means the hydrogel is non-Newtonian fluid as well as shear thinning.

Assuming the geometry of the printed hydrogel strand is constant from the start point to the end point and the strand is printed out in a continuous manner, the flow rate $\dot{Q}$ is equal to

$$\dot{Q} = \frac{\pi d^2}{4} \dot{v} \quad (3)$$

where d is the printed strand diameter and $\dot{v}$ is the stage moving speed (m/s).

If we substitute $\dot{Q}$ in Equation (3) into Equation (2), we obtain

$$\dot{\gamma}_w = \frac{3n + 1}{4n} \cdot \frac{32\dot{Q}}{\pi D^3} = \frac{3n + 1}{4n} \cdot \frac{8d^2\dot{v}}{D^3} \quad (4)$$

From apparent viscosity, we can find the relationship between stress and shear rate, which is

$$\tau_w = \eta \dot{\gamma}_w \quad (5)$$

If we substitute τ_w from equation (1) and $\dot{\gamma}_w$ from equation (4) into Equation (5), we obtain

$$\frac{D\Delta P}{4L} = \eta \cdot \left(\frac{3n + 1}{4n} \cdot \frac{8d^2\dot{v}}{D^3} \right)$$

Rearrange the above equation, we obtained.

$$\Delta P = 32\eta L \left(\frac{3n + 1}{4n} \right) \cdot \frac{d^2}{D^4} \cdot \dot{v} \quad (6)$$

Therefore,

$$d^2 = \frac{D^4}{32\eta L \dot{v}} \cdot \left(\frac{4n}{3n+1} \right) \cdot \Delta P \quad (7)$$

or

$$d(\dot{v}, \Delta P) = D^2 \cdot \sqrt{\frac{1}{32\eta L \dot{v}} \cdot \left(\frac{4n}{3n+1} \right) \cdot \Delta P} \quad (8)$$

where ΔP is the gauge pressure (Pa).

Thus, the relation between printed hydrogel width (resolution, d), pressure (ΔP), stage moving speed ($\dot{v}$), and nozzle size (D) can be described as shown below.

At a constant nozzle size: $d \propto \sqrt{\frac{1}{\dot{v}}}$ and $d \propto \sqrt{\Delta P}$ or

At a different nozzle size:

$$d \propto D^2 \sqrt{\frac{1}{\dot{v}}} \text{ and } d \propto D^2 \sqrt{\Delta P}$$

Table 3.1 shows the constants that are used for verifying Equation (8). The power law index and apparent viscosity were determined from rheological study, which will be described in section 3.4. The needle length was measured from the needle purchased from Nordson EFD (East Providence, RI), and the atmospheric pressure is at standard pressure.

Table 3.1. Constant values for mathematical model verification.

Pluronic Concentration	24.5 wt%	30 wt%
Power law index (n)	0.0511	0.1656
Apparent viscosity (η)	1.04 Pa.s	1.46 Pa.s
Needle length (L)	0.005 m	0.005 m

3.3. Materials and methods

3.3.1. Pluronic F127 hydrogel preparation

Pluronic F127 (P2443) was purchased from Sigma-Aldrich, USA. Pluronic F127 is in white powder form. In order to make 24.5 wt% pluronic hydrogel, 12.25 g of pluronic were mixed with 50 ml of deionized (DI) water under vigorous stirring at 4 °C. For 30 wt% pluronic hydrogel, 15 g of pluronic were mixed with 50 ml DI water under vigorous stirring at 4 °C. After the solution was mixed and became homogeneous, 5 ml of pluronic hydrogel was poured into a syringe before the actual rheological testing and printing.

3.3.2. Rheological characterization

In order to develop the pressure model, a rheological study was conducted at first. A 40-mm, 2.022° cone plate rheometer (DHR, TA Instruments) was used to obtain the power law index (n) of the prepared pluronic F127 hydrogel. From the work of Abdel-Hamid *et al.* (2006), the power law model was the best fit for pluronic compared to the Bingham and Casson model [227]. Thus, in this work, we chose the power law model for the rheological study which showed a good fit at $R^2 = 0.99624$ as shown in Figure 3.2. When the reference shear rate ($\dot{\gamma}_0$) = 1 s^{-1} , the relationship of viscosity, shear rate and power law index can be described in Equation (9) below [225]:

$$\eta = \eta_0(\dot{\gamma})^{n-1} \quad (9)$$

where η is apparent viscosity, η_0 is zero viscosity and $\dot{\gamma}$ is shear rate. In order to find the power law index (n), both sides of Equation (3.9) were changed into $\log \dot{\gamma}$ form to obtain a linear region.

$$\log \eta = (n-1)\log \dot{\gamma} + \log \eta_0 \quad (10)$$

From the rheometer, we obtained the relationship of viscosity and shear rate for 24.5 wt % pluronic hydrogel. As shown in Figure 3.2a, pluronic viscosity decreases when shear rate increases, which shows that pluronic is a shear thinning polymer, and the power law index (n) can be found from curve fitting in Figure 3.2b at the shear rate that was in a linear region from starting point to 1500 s⁻¹ shear rate, in which n = 0.0511.

3.3.3. Apparent viscosity (η) calculation

In order to minimize the number of printing variables, firstly, the material's viscosity need to be determined which is considered constant and has been used for the theoretical model.

The shear rate in capillary of power law flow ($\dot{\gamma}_w$) can be found equation (4) as mentioned earlier in section 3.2. which are

$$\dot{\gamma}_w = \frac{3n+1}{4n} \cdot \frac{8d^2\dot{v}}{D^3} \quad (4)$$

Assume that at $v = 0.03$ m/s, there is no change in hydrogel geometry and $d = \frac{D}{2}$, therefore

$$\dot{\gamma}_w = \frac{3n+1}{4n} \cdot \frac{8\left(\frac{D}{2}\right)^2\dot{v}}{D^3} = \frac{3n+1}{4n} \cdot \frac{8(D)^2\dot{v}}{4D^3}$$

After deduction we obtained,

$$\dot{\gamma}_w = \frac{3n+1}{4n} \cdot \frac{2\dot{v}}{D} \quad (11)$$

Nozzle size 21G, $D = 0.000514$ m and n is power law index (n = 0.0511).

Substitute D, n and $\dot{v}$ into the equation (11)

$$\dot{\gamma}_w = 658.64 \text{ s}^{-1} \text{ or } \sim 660 \text{ s}^{-1}$$

At the shear rate of 660 s^{-1} , apparent viscosity (η) is around 1.04 Pa.s as shown in Figure 3.2 below. The viscosity for 30 wt% pluronic was calculated by using the same method.

Figure 3.2. a) Viscosity vs shear rate cure with curve fitting ($R^2 = 0.99624$) and b) viscosity and shear rate in a linear region for curve fitting, (yellow region in log-log scale).

3.3.4. Resolution study based on three different factors

3.3.4.1. Pluronic F127 hydrogel Printing

For 24.5 wt% pluronic, three different nozzle sizes were used, 21G (inner diameter: $514 \mu\text{m}$), 25G (inner diameter: $260 \mu\text{m}$) and 27G (inner diameter: $210 \mu\text{m}$). Three different levels of pressure ($1, 2, \text{ and } 3 \times 10^5 \text{ Pa}$) and three levels of

stage moving speed (0.01, 0.02, and 0.03 m/s) were studied in this printing experiment by using a pneumatic extrusion-based bioprinter (Regenhu, Villaz-St-Pierre, Switzerland). A total of 27 ($3 \times 3 \times 3$) experiments were investigated. Hydrogel was printed onto a clear glass slide, which is the platform for holding the structure. For 30% pluronic, two different nozzle sizes were used: 21G and 25G. The stage moving speed at 0.03 m/s and six different pressure conditions were studied for model verification. ΔP and D values are presented in Table 3.2 and Table 3.3.

Table 3.2. Gauge pressure (ΔP) and Nozzle diameter (D) value used in equation 10 for 24.5 wt % Pluronic.

	Needle diameter, D (m)	Gauge pressure, $\Delta P \times 10^5$ (Pa)		
		Stage moving speed		
		0.01 m/s	0.02 m/s	0.03 m/s
21G	0.000514	1.26	1.52	1.80
25G	0.000260	2.50	3.00	3.60
27G	0.000210	3.10	3.71	4.46

Table 3.3. Gauge pressure (ΔP) and Nozzle diameter (D) value used in equation 10 for 30 wt% Pluronic.

	Needle diameter, D (m)	Gauge pressure, $\Delta P \times 10^5$ (Pa)		
		Stage moving speed		
		0.01 m/s	0.02 m/s	0.03 m/s
21G	0.000514	1.77	2.12	2.50
25G	0.000260	3.50	4.20	5.04
27G	0.000210	4.33	5.20	6.24

3.3.4.2. Pluronic F127 hydrogel Characterization

The widths of the printed lines in all 27 experiments were measured by using an optical microscope (Carl Zeiss, Oberkochen, Germany) at 5x magnification. In each experiment, three lines were printed continuously.

3.4. Results and discussion

3.4.1. Resolution study on a pneumatic extrusion-based bioprinter

Table 3.4 shows the line width of printed pluronic hydrogel strands at various nozzle sizes, printing speeds and gauge pressures. From the results, it can be seen that, in order to get a continuous line, the pressure must be sufficient to dispense a continuous strand at the given speed. However, when there is too much pressure (at operation pressure 3×10^5 Pa of 21G nozzle size), it is obvious that the width of hydrogel strand becomes larger than the nozzle size (514 μm). Similarly, the results of 25G show that hydrogels can be printed continuously at 3×10^5 Pa, but the pressure is too high, leading to a width larger than the nozzle size (260 μm), as shown in Figure 3.3a. Surprisingly, the line width result from 27G nozzle at 3×10^5 Pa with a 0.01 m/s speed is also wider than the nozzle size. This is possibly due to the fact that pluronic was printed in discrete elongated droplets instead of a continuous strand, as shown in Figure 3.3b. The discontinuous strand may result from the surface tension that becomes dominant after extrusion. However, in this experiment, pluronic F127 at 24.5% w/v was used, which is a high-viscosity hydrogel (1.04 Pa.s or 1040 cP). Hydrogel that has a high viscosity may be able to overcome the effect of surface tension [228]. Therefore, for the mathematical modelling, this phenomenon will be negligible due to the fact that it is not significant to be included in the model. These elongated droplets are typically wider than a straight line.

Table 3.4. Line width results from extrusion-based bioprinter in micrometers (μm)

21G (514 μm)		Gauge pressure x 10^5 (Pa)		
	Stage moving speed (m/s)	1	2	3
	0.01	566.17 $\pm$ 14	2668.05 $\pm$ 10	3215.85 $\pm$ 24
	0.02	368.75 $\pm$ 7 ^b	1771.66 $\pm$ 30	2237.90 $\pm$ 20
	0.03	325.48 $\pm$ 2 ^b	1512.28 $\pm$ 7	1852.55 $\pm$ 15
25G (260 μm)		Pressure x 10^5 (Pa)		
	Stage moving speed (m/s)	1	2	3
	0.01	N/A ^a	325.06 $\pm$ 17 ^b	1447.95 $\pm$ 1
	0.02	N/A ^a	286.72 $\pm$ 33 ^b	527.68 $\pm$ 19 ^b
	0.03	N/A ^a	220.72 $\pm$ 23 ^b	406.20 $\pm$ 13 ^b
27G (210 μm)		Pressure x 10^5 (Pa)		
	Stage moving speed (m/s)	1	2	3
	0.01	N/A ^a	254.69 $\pm$ 9 ^b	435.47 $\pm$ 7 ^b
	0.02	N/A ^a	N/A ^a	385.46 $\pm$ 11 ^b
	0.03	N/A ^a	N/A ^a	281.86 $\pm$ 18 ^b

^aNo hydrogel was printed out (not sufficient applied pressure). ^bNot continuous strand (defect strand).

Another interesting observation is that, at a higher stage moving speed, the printed hydrogel has more defects compared to the hydrogel line that was printed at a lower speed, as shown in Figure 3.3c and 3.3d. By using a 21G nozzle at the highest stage moving speed, the thinnest line can be fabricated. This is similar to the finding from the work of Müller *et al.* who used a micro-valve-based nozzle to print pluronic [104]. Even though a finer line has been obtained, it was not suitable to print a complex structure at this speed (0.03 m/s). The defects in the first layer will not allow the build-up of a 3D structure. Rather, a moving speed

of 0.02 m/s is more suitable to print a continuous uniform hydrogel strand (Figure 3.3c).

Figure 3.3. Pluronic hydrogel printed strand under 5x magnification optical microscope at **a)** 25G nozzle, 3×10^5 Pa, 0.01 m/s speed, **b)** 27G nozzle, 3×10^5 Pa, 0.01 m/s speed, **c)** 21G nozzle, 1×10^5 Pa, 0.02 m/s speed and **d)** 21G nozzle, 1×10^5 Pa, 0.03 m/s speed, where the red arrows pointed to the line defects (scale bar = 200 μ m).

3.4.2. Model verification

Dispensing pluronic F127 hydrogel with different nozzle sizes on the pneumatic extrusion bioprinter at different stage moving speeds ($\dot{v}$) produces different results. As shown in Figure 3.4, both theoretical and experimental line widths that represent resolution were plotted. It shows the relationship between the pressure and the hydrogel strand width of the model and the theoretical data at different stage moving speeds. The aforementioned model was developed with the following assumptions: (i) no slip at wall; (ii) fluid is incompressible; (iii)

flow is steady and laminar; (iv) no change in strand cross sectional surface; and (v) the pressure drop at the entrance and exit are negligible.

Figure 3.4. Relationship of d vs. $D^2\sqrt{\Delta P}$ of experimental data and theoretical data at different condition: **a)** $\dot{v} = 0.01 \text{ m/s}$, **b)** $\dot{v} = 0.02 \text{ m/s}$ and **c)** $\dot{v} = 0.03 \text{ m/s}$.

This model does not include the effect of temperature and degree of printed strand continuity. For a speed of $\dot{v} = 0.01 \text{ m/s}$, some experimental data are close to the theoretical data, but the off data points made the overall trend differed from the model (Figure 3.4a). On the other hand, as shown in Figure 3.4b and 3.4c, the experimental data from $\dot{v} = 0.02 \text{ m/s}$ and $\dot{v} = 0.03 \text{ m/s}$ have some values that stay in the region between the predicted models. Nevertheless, they still show similar

trends compared to the theoretical data. Figure 3.4 shows the experimental data following the model trend, which is $d \propto D^2\sqrt{\Delta P}$.

Even though pluronic can gel at 20 wt%, due to the micelle packing network, pluronic is commonly printed at 24.5 wt% or higher [68, 101]. Thus, two concentrations of pluronic (24.5 wt% and 30 wt%) were used and compared. As shown in Figure 3.5, this model proved that it can work with different concentrations. The data points followed the model trend, though some errors occurred. In a future work, the correction factor should be included to minimize this error.

Figure 3.5. Relationship of d vs. $D^2\sqrt{\Delta P}$ of experimental data and theoretical data at different concentrations at $\dot{v} = 0.03 \text{ m/s}$: **a)** 24.5 wt% pluronic and **b)** 30 wt% pluronic.

Though the theoretical results show similar trends, they still show some errors. This error may be due to the change in the effect of pressure during entrance to, and exit from, the nozzle. Moreover, there is a change in pressure from the syringe barrel to the nozzle, which may affect the results as well. As shown in Figure 3.4, the error is reduced at a higher speed, which may be due to the fact

that this bioprinter is a pneumatic system and it always has a pressure drop [228], which results in material returning to its original viscosity, owing to the thixotropic properties of materials [229]. However, at a higher speed, less time is needed to allow material to recover. Therefore, viscosity is constant at higher speeds. Moreover, the die swell of hydrogel might be another reason for the error between actual results and the model [230]. However, Feilden *et al.* (2016) showed that pluronic has shear thinning properties, which eliminates the die swell problem and allows the filament to emerge smoothly [231]. Therefore, in this work, the die swell was not included in the model. Nonetheless, in the future, the die swell may be added as one of the correction factor for the hydrogel that shearing at slow speed. Additionally, another equation proposed by Zhong et al. [232] which suitable for high viscosity polymer may be an alternative to provide a better results from this model. Lastly, the change in geometry of the hydrogel strand upon its exit from the nozzle may also contribute to the error, as one study reported that the deviation could be due to the internal diameter of the nozzles (needle) lack of uniformity [232, 233]. A correction factor may be added into the model to eliminate this error.

The objective of this chapter is to develop a simple yet accurate mathematical model to predict the line width of hydrogel strand at a given speed, the minimum gauge pressure required and the nozzle size. Moreover, by understanding how each parameter influences the width of dispensed hydrogel in a pneumatic-based bioprinter system, an optimum condition can be found to obtain a complex hydrogel structure with good resolution, as shown in Figure 3.6 (at optimum condition, grid pattern at different spacing from 2 mm to 0.5 mm (from left to right) can be fabricated. However, this model is unable to predict and incorporate

the sol-gel transition behaviour of thermos-responsive hydrogels, which have properties that affect viscosity of the hydrogels. In order to investigate the hydrogel properties effect on printability, the nozzle size should be kept constant to deeply explore significant effects. In fact, pluronic F127 has a good printability at a range of viscosities [101, 104]. Although synthetic polymers are relatively easy to print and can be biocompatible [234], they are usually not bioactive and may not be suitable for long-term cell culture [59]. Recently, Kolesky *et al.* showed that, by extruding pluronic as a sacrificial mould, gelatin methacrylate (GelMA) constructs with micro-channels can be fabricated, which could be potentially useful for vascularized tissue engineering [62]. Nevertheless, the resolution achievable from pneumatic extrusion-based bioprinter still remains elusive [235].

Figure 3.6. Grid structure (single layer) of pluronic F127 by using 21G nozzle at 1×10^5 Pa and 0.025 m/s speed (scale bar = 5 mm).

In this research, we modelled the relationship of extrusion-based bioprinting parameters by using thermo-responsive hydrogel pluronic F127. Pluronic F127 is the hydrogel that the resolution can be determined directly after printing. Other hydrogels, for example alginate, is crosslinked by an ionic crosslink reaction. The final resolution still depends on the swelling after the alginate reacts with a divalent ion such as Ca^{2+} and Ba^{2+} [236]. On the other hand, GelMA is thermo-responsive and a UV-crosslinked hydrogel, and the final resolution of GelMA

can only be determined after a UV curing process. This process may lead to shrinkage of the GelMA hydrogel strand [237]. In this work, the gelation mechanism and the effect of post-processing on the hydrogel strand were not included in the model. Hence, this analytical model needs to be further developed in order to be applied to other types of thermo-responsive hydrogels or even cell-laden hydrogel, which have been used extensively for tissue engineering and other biomedical applications, for predicting optimal processing conditions.

3.5. Summary

A simple yet accurate analytical model has been developed for predicting the resolution of extrusion bioprinting. Pluronic F-127 at 24.5 wt% was printed by using a pneumatic extrusion-based bioprinter and the width of printed hydrogel strand was measured against the size of the nozzle, pressure and stage moving speed. Experimental data showed a good agreement with model predictions, despite some errors possibly due to the capillary entrance and hydrogel surface tension effects but at the high stage moving speed, this error seemed to be negligible. The knowledge obtained from this research might help develop new pluronic-based bio-ink formation and optimize bioprinting parameters of new bio-ink which will be further described in Chapter 4.

Chapter 4 Hydrogel composite synthesis and characterization

4.1. Introduction

As mentioned earlier, vascularization is a major problem in tissue engineering (TE), especially for engineering thick tissues measuring more than 200 μm . Insufficient vascularization limits the oxygen and nutrient transfer to cells, which can lead to hypoxia and formation of non-uniform tissue structures [6-8]. Vasculature-like structures are usually multilayer [238] and hollow [239]. They also contain curvature parts and size variations to connect through the body system. Thus, it is quite difficult to imitate this complex 3D structure using materials similar to native vessels such as hydrogels. Though a few techniques have been explored to fabricate this complex 3D structure, direct bioprinting of such a complex 3D structure on a non-liquid platform in a single step has not been realised [62, 118, 240].

In order to create complex 3D hydrogel structures, a few researches have explored the feasibility of printing two dissimilar hydrogels in micro-extrusion process. One hydrogel serves as support or sacrificial material and the other for physical modelling. For example, Bertassoni *et al.* used agarose strand as a mould to create patterns on UV crosslinked hydrogel structures by embedding it inside the hydrogel block before it was crosslinked [88]. Kolesky *et al.* used pluronic F127 as sacrificial materials and created hollow channels in GelMA for vascularized tissue structures [62]. Recently, a new technique named “FRESH” used gelatin (at controlled temperature and specific to the type of model materials) as support and other hydrogels such as alginate, collagen and fibrin as model materials for fabricating complex 3D hollow structures [79]. Collectively, these

researches suggest that, in order to create a 3D complex hydrogel structure, the use of sacrificial material or support materials is unavoidable. However, the reported techniques are dependent on a liquid platform and unable to directly print both model hydrogel and support hydrogel on a non-liquid platform such as wounds. Moreover, they are unable to print at room temperature or human body temperature, mainly because the number of model materials that have both good printability and good shape fidelity at these temperatures are very limited [241]. In this chapter, a new hydrogel composite that provides printability, shape integrity and biocompatibility for fabricating complex 3D structures in a single step without relying on any liquid platform is reported. The highly printable hydrogel composite was designed and synthesized from pluronic 127 and GelMA. Rheological properties and water swelling properties of the hydrogel composite were investigated. After that, the hydrogel composite was printed as a model material along with pluronic 127, a support material, to achieve a perfusable vasculature-like structure.

4.2. Materials and methods

4.2.1. Pluronic-GelMA (Plu-GelMA) hydrogel composite synthesis

Pluronic chain was modified by reacting with succinic anhydride to form pluronic monocarboxylate (Plu-MP) similar to the method described by Park *et al.* [242]. As shown in Figure 4.1a, firstly, pluronic F127 was dissolved in Dioxane with 4-Dimethylaminopyridine (DMAP) and Triethanolamine (TEA) as a catalyst. The reaction was run under vigorous stirring condition for 24 hours. Next, the Dioxane was removed and the Plu-MP powder was precipitated by washing with Diethyl-ether and dried in vacuum oven overnight to get rid of the organic compounds. Then, purified Plu-MP powder was mixed with Phosphate buffer

saline (PBS) to make 30 wt% Plu-MP hydrogel. The hydrogel was later stored at 4 °C before further synthesis and testing.

Gelatin methacrylate (GelMA) was fabricated by reaction between methacrylate anhydride and gelatin at 50 °C in PBS as shown in Figure 4.1b similar to the method that previously described by Narbat *et al.* [243].

Figure 4.1. Schematic of chemical reaction for **a)** pluronic monocarboxylate (Plu-Mp) and **b)** gelatin methacrylate (GelMA).

The reaction was run for two hours under constant stirring condition. After that, the reaction was stopped by diluting the solution fivefold with PBS. The diluted solution was further dialyzed with Deionized water by using 12-14 kDA molecular weight cutoff (MWCO) dialysis tubes for 1 week. Next, the GelMA was frozen overnight at -30 °C, lyophilized for 5-7 days, and stored at -30 °C. GelMA was prepared by mixing freeze-dried GelMA foam at concentrations of 15 wt% and 0.2 wt% of the photo-initiator (2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone, Irgacure 2959) in PBS. GelMA with photo-initiator was kept in the dark at 37 °C before mixing to prevent gelation.

Finally, Plu-MP was mixed with GelMA as shown in Figure 4.2a by putting two of them into two syringes; 15% GelMA with 0.2% of Irgacure 2959 and 30% Plu-MP at varying mass ratio. Then, the two syringes were connected using a 3-way stopcock (Discofix® C, B.Braun) as shown in Figure 4.2b. The materials in both syringes were mixed by extruding them into another syringe. After that, the syringe with hydrogel composite was wrapped with aluminium foil and kept at room temperature overnight for the reaction to complete. The hydrogel composites at different mass ratio of Plu-MP:GelMA were casted or printed in different shapes followed by UV crosslinking for 120 seconds by using UV flood curing system (Techno Digm, Singapore) before further characterization (except NMR). All the chemicals in this work were purchased from Sigma-Aldrich unless mentioned elsewhere.

Figure 4.2. a) Schematic of chemical reaction for synthesis Pluronic-GelMA composite and b) Hydrogel mixing by using 3-way stopcock.

4.2.2. Chemical characterization

For ^1H -Nuclear magnetic resonance spectroscopy (^1H -NMR), the reactants and synthesized polymers were characterized by ^1H -NMR 400 MHz, (AVANCE I, Bruker, Germany) in D_2O solvent (Sigma-Aldrich, USA) of the peaks at 1.8–2.0, 5.7–5.9, 6.1–6.3 ppm from methacrylate group and the peaks at 1.0-1.2, 3.5-3.7 ppm from Plu-MP functional group. For attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) study, UV-crosslinked hydrogel samples were characterized in ATR mode by using Thermo Scientific Nicolet™ 6700 FT-IR spectrometer (Cambridge, UK) which was equipped with OMNIC software. Samples were mounted onto the orbit sampler. The spectra's results were demonstrated in the range of $500 - 4000 \text{ cm}^{-1}$ with a resolution of 4 cm^{-1} .

4.2.3. Mechanical tests

Mechanical properties of the hydrogel composite samples were tested at room temperature using a Model 5566 Instron universal testing machine (Instron, Norwood, MA, USA). The hydrogel samples of each concentration were prepared for both compression and tensile tests. The dog bone shaped samples ($n = 3$, $10 \times 15 \text{ mm}$) were prepared for tensile test while the circular dishes ($n = 3$, $\varnothing = 10 \text{ mm}$) were prepared for compression test. Loads of 100 N and strain rate of 1 mm/min were applied.

4.2.4. Microstructure and morphology analysis

For primary fixation, 2.5 % (v/v) glutaraldehyde solution (Sigma-Aldrich, USA) was used. All samples were soaked in 2.5% glutaraldehyde solution for 1 hour at 4°C . After that, samples were washed with distilled water for several times to remove glutaraldehyde. Next, ethanol was used to dehydrate the cells by a series of concentrations (v/v): 25%, 50%, 70%, 95%, 100% and 100%. Samples were

soaked in each ethanol concentration for 10 minutes. After that, samples were washed with distilled water and dried in a desiccator for 1 day. Next, the hydrogel samples were coated with gold at 10 mA for 20 seconds before the SEM examination.

4.2.5. Rheological test

A rheological study was conducted with each concentration of Plu-GelMA hydrogel composite by using a 40-mm, parallel plate rheometer (DHR, TA Instruments). The temperature was kept constant at room temperature throughout the experiment. The experiment was run at shear rate $1 - 5000 \text{ s}^{-1}$ in order to obtain a rheological profile of the hydrogel composite.

4.2.6. Swelling test

Hydrogel composite samples were casted into circular disc ($n = 3$, $\varnothing = 10 \text{ mm}$, height = 5 mm). After that, samples were dried by using freeze dryer and kept at -80°C before further experiments. Subsequently, hydrogel samples were soaked in DI water for 4 hours to ensure that they were fully swollen and the swell ratio of hydrogel was calculated by using formula in equation (1) below

$$\text{Swelling ratio} = \frac{W_{\text{swollen}} - W_{\text{dry}}}{W_{\text{dry}}} \times 100 \% \quad (1)$$

4.2.7. Diffusion test

The FITC-dextran (4 kDa, Sigma) solution at concentration levels of 0.0001, 0.0005, 0.001, 0.005, 0.01 and 0.05 mg/ml were analysed with microplate reader (SPARK 10M, Tecan) at excitation 485 nm, emission 535 nm for generating standard curve. The results and curve fitting are shown in Appendix A.

In order to test the porosity level of each hydrogel composite concentration, the diffusion test is similar to the FITC-dextran loading and releasing that was used by Martin *et al.* [244]. Briefly, Dried composite hydrogels ($\approx 40 \text{ mg}$, $n > 3$) were

dispersed in FITC-dextran (4 kDa, Sigma) solutions at concentration of 2.0 mg/ml into dried hydrogels. After shaking for homogeneous absorption for two hours, the hydrogel samples were placed in PBS followed by shaking to ensure equal diffusion. The supernatant (PBS solution with FITC-dextran loaded sample) was taken out every hour from 0-4 hours to check the amount of FITC-dextran diffused. The supernatant was later analysed with microplate reader (SPARK 10M, Tecan at excitation 485 nm, emission 535 nm).

4.2.8. Printability test and printing of 3D complex structure

The cylindrical CAD file was designed by using BioCAD™. The STL files were sliced by using the STL converter program. Both programmes were attached with a pneumatic extrusion-based bioprinter (Regenhu, Villaz-St-Pierre, Switzerland). The hydrogel composites were loaded in 5 ml syringe before printing. The printing condition of all concentrations was as follows: stage moving speed of 500 mm/min, pressure of 3-5 bar (depends on the composite ratio), temperature at 30±3 °C and nozzle of 27G (inner diameter = 210 µm). 24.5% Pluronic F127 was used as support material. After printing, the hydrogel samples were cured by using UV flood curing system (Techno Digm, Singapore) for 120 seconds and soaked in cold water (4 °C) for 2-3 hours before further experiment. Later the hollow hydrogel structures were taken for perfusion tests using red dyed liquid and air to further investigate on perfusion property.

4.2.9. Statistical analysis

The statistical significance was determined by Student t-test study for two groups of data or analysis of variance. P-values were presented as statistically significant and highly significant as *P < 0.05 (at 95% level of confidence), ** P < 0.05 is for these significantly different from the rest.

4.3. Results and discussion

4.3.1. Synthesis and characterization of Pluronic-GelMA (Plu-GelMA) hydrogel composite

The synthesis started with Plu-MP and GelMA hydrogel (chemical structure of both compounds were shown in Figures 4.1a and 4.1b) in liquid state at different mass ratios (Plu-MP:GelMA = 1:2, 1:1.5, 1:1, 1.5:1 and 1:2) by using a 3-way stopcock connector. As shown in Figure 4.3b the reaction started upon mixing Plu-MP micelles with GelMA un-coiled chains to form a weak physical bond between -COO^- group and -CONH_2^+ (as shown in Figure 4.3a)

Figure 4.3. **a)** Structure and functional group of reactants and **b)** Schematic of Plu-GelMA synthesis (top) and fabrication process (bottom).

After mixing, the samples were left overnight at room temperature, to allow the hydrogel to completely shift from solution (liquid) state into homogeneous and translucent gel (solid) state. Next, as shown in Figure 4.3b, the hydrogel composite was used for casting or printing, followed by the UV exposure to generate photo-crosslinked chemical bonds in the hydrogel structure. The unreacted Plu-MP was later washed away by using cold water.

In this research, a highly printable hydrogel composite has been successfully developed. By modifying pluronic into Plu-MP, the hydrogel composite is able

to stay in single phase due to physical ionic bond between carboxylic group and amine group. Moreover, by using a three-way stopcock, the energy was added into the mixture similar to the use of homogenizer which might lead to the formation of stable foam structure inside the hydrogel [105]. The UV crosslinking process allows the methacrylate inside the GelMA to be dominant and improves the shape integrity of the printed structures. However, unlike the grafting reaction by EDC/NHS coupling [105, 242], by using this technique, some of the pluronic did not react but bind with GelMA. Thus, the use of cold water was required in order to eliminate unreacted part which might affect cell viability during cell culture. This is because pluronic is not able to provide mechanical strength to support cell adhesion due to physical micelles packing gelation [55, 148].

4.3.2. Chemical characterization

The NMR results showed that Plu-MP and GelMA were different from their composite Plu-GelMA due to the mixing and overnight reaction. The zoom-in image in Figure 4.4a shows that the methacrylate functional group was present in Plu-GelMA, making the thermo-responsive composite also photo-crosslinkable. The FTIR results in Figure 4.4b confirm that more GelMA contents can lead to more hydrophilic O-H functional groups (free water hydroxyl groups) in $3200\text{-}3600\text{ cm}^{-1}$ [245, 246], hence this might have resulted in a higher water swelling ratio.

Figure 4.4. Properties of Plu-GelMA composite at different mass ratio: **a)** NMR results of GelMA, Plu-MP and Plu-GelMA, the red arrows pointed the functional group that influenced from GelMA and **b)** ATR-FTIR results of UV crosslinked Plu-GelMA at different ratio.

4.3.3. Mechanical tests

For tensile test as shown in Figure 4.5a, there was a significant drop of tensile modulus (Young's modulus) at higher Plu-GelMA ratios. At ratio 1:2 (Plu-MP:GelMA), the tensile modulus was around 93 ± 10.1 kPa and drastically dropped to 57 ± 5.2 kPa when the ratio changed to 1:1. Lastly, for 2:1 ratio, the tensile modulus dropped to 14.6 ± 2.7 kPa. This is expected due to the fact that GelMA is the part that gives mechanical strength owing to their chemical crosslinked bonds [247]. On the other hand, for tensile strain as shown in Figure 4.5b, the 2:1 Plu-GelMA seems to be more elastic due to a strain value of 0.64 ± 0.16 mm/mm. However, there is no statistically significant difference in the mean values for all ratios at 95% confidence level.

Figure 4.5. Mechanical testing of Plu-GelMA composite at different mass ratio: **a)** Tensile modulus, **b)** tensile strain, **c)** compressive modulus and **d)** compressive strain ($n \geq 3$, statistical significance determined by pairwise t-test where $*P < 0.05$).

For compression test, at 95% level of confidence, there is also no statistical difference in terms of compressive modulus and compressive strain for all Plu-GelMA ratios. However, the highest compressive modulus was from 1:2 Plu-GelMA which was around 102.7 ± 21.6 kPa as shown in Figure 4.5c. On the other hand, for compressive strain, the results were around $0.35\text{--}0.47 \pm 0.15$ mm/mm for all the ratios. The fluctuation in the compressive strain values as shown in Figure 4.5d might result from random porous structures inside the samples. However, the result of compressive strain for each ratio is relatively similar and there is no statistically significant difference. For both tensile and compression tests, the amount of GelMA content plays a significant role in the mechanical strength.

4.3.4. Microstructure and morphology of hydrogel composite

The microstructure of Plu-GelMA hydrogels composite are shown in Figure 4.6. The SEM images show that more pluronic content led to larger interconnected pores. Similar work has been investigated by Saraiva *et al.*, when GelMA was integrated with other hydrogels, reduced content of GelMA led to smaller pore structure [248]. The diffusion ability of hydrogel composite was later investigated by using FITC-dextran as described in the next section.

Figure 4.6. SEM images at 500x magnification of Plu-GelMA composite at different ratio: a) 1:2, b) 1:1 and c) 2:1.

4.3.5. Rheological, swelling and diffusion properties of hydrogel composite

The rheological properties of Plu-GelMA hydrogel composite are shown in Figure 4.7a. All the hydrogels showed a shear thinning behaviour which was similar to pluronic and GelMA [239, 249]. At the high shear rate region (shear rate beyond 100 s^{-1}), the viscosities of all hydrogel composites dropped drastically. At a higher content of pluronic, especially at the ratio of 2:1, the overall shape of the curve looks more linear than at other ratios. As mentioned earlier in the chemical characterization section in 4.3.2., the presence of the free

water hydroxyl groups in 3200-3600 cm^{-1} [245, 246] resulted in higher water swelling ratio (as shown Figure 4.7b). The GelMA content in the composite affected water swelling properties, and higher GelMA content led to lower water absorbability. Additionally, the strong covalent bonds formed from photo-crosslinking reaction might also contribute to lower swelling ratio [61, 247]. Moreover, when there was more pluronic in the composite (e.g. Plu-GelMA 2:1), the pore structure was larger (as shown in Figure 4.6c). This might also contribute to the swelling ratio as a larger pore structure allows more water to be absorbed [248]. The diffusion test results in Figure 4.7c showed that the hydrogel composite was porous and Plu-GelMA ratio 2:1 showed the best efficiency of FITC release. This could result from the micro-porous structure because at this ratio the pore structure was more obvious and larger than at other ratios.

Figure 4.7. a) Plu-GelMA rheological study of viscosity vs shear rate, b) water swelling ratio of Plu-GelMA hydrogel composite at different ratio ($n = 3$, statistical significance determined by pairwise t-test where $*P < 0.05$) and c) diffusion test by using a FITC-dextran release of different concentrations of hydrogel composite.

4.3.6. Printability and perfusability of 3D complex structure

The printing process flow diagram is shown in Figure 4.8a. For a simple hollow cylindrical structure (5 layers and 50 layers), support material was not required. Plu-GelMA at a ratio of 2:1 could be used directly for single-step printing of these simple structures. Hydrogel composites at other mass ratios were also able to print but at a lower height only (less than 50 layers) as shown in Figure 4.8d. The printability of Plu-GelMA is dependent on pluronic contents, the more pluronic, the better printability. This is because pluronic has micelles packing which is easy to flow and structurally stable at a wide range of temperatures (from 20 – 40 °C) [98, 148]. Therefore, the Plu-GelMA 2:1 was selected to be the model materials for printing 3D complex structures. However, beyond the ratio of 2:1 was not considered because it made GelMA too low in concentration to be firmly cured later. Thus, at the higher Plu-MP ratio more than 2:1, the printed part will just disappear after being soaked in water and losing all the mechanical strength. Moreover, Plu-GelMA 2:1 is stable at a wide range of temperature similar to pluronic, thus printing at cell favourable environment such as human body temperature is possible. However, the concentration of pluronic in model material and support material must be close enough to avoid material interaction and diffusion due to the difference in osmotic pressure [241].

Figure 4.8. **a)** Process diagram of 3D complex structure fabrication, **b)** Schematic of printing process, blue ink represents model material and red ink represents support material, **c)** Hollow vascular branch structure printing from STL file to actual part in water and **d)** printability test of Plu-GelMA hydrogel composite at 5 layers and 50 layers (scale bars, 5 mm).

In order to fabricate complex structures, support material and dual-nozzle printing must be used. The printing process is shown in Figure 4.8b, in which one nozzle was used to print Plu-GelMA as model material and the other to print Pluronic as support material. Both model and support materials were printed using the same processing parameters, and good printability and repeatability were achieved (Figure 4.8c). After printing, the parts were UV cured and washed in cold water to remove support materials. The patency of the hollow structure could be easily observed when the parts were immersed in the water (Figure 4.8c, side view).

Not only that the Y-shaped vascular tubes in Figure 4.8c could be printed, but also the 3D quadfurcated structure could be fabricated (Figure 4.9a). The 3D quadfurcated vasculature-like structure was fabricated by using Plu-GelMA 2:1 as model material and 24.5 wt% Pluronic as support material on a solid platform in a single step printing. In order to test the perfusability of the hollow structure, a red dye was first purged into the structure, followed by an air perfusion test. The structure turned red because of the inflow of the red dye. A moving stream of red liquid was clearly visible at the exit of the quadfurcated structure. Likewise, air bubbles were seen continuously exiting from the opening. Figure 4.9b and 4.9c showed that this soft vasculature-like structure is perusable to both liquid and gas. The liquid and gas perfusion tests proved that the 3D structure was patent and perfusable. However, some of the liquid ink was absorbed into the structure at the initial of the test due to swelling and porosity of the hydrogel.

Figure 4.9. Perfusion study of a 3D quadfurcated vasculature-like structure: **a)** 3D complex hollow structure fabrication from STL file to actual part in water (red arrows point the hollow part), **b)** Liquid perfusion test (yellow arrow points liquid that came out) and **c)** air perfusion test (yellow arrow points air bubbles that came out).

4.4. Summary

The 3D complex hollow structures are desirable for many applications, especially for vascularized tissue engineering or even tissue models for drug testing. Nowadays, limited options are available for fabricating soft 3D complex hollow structures directly on a solid platform in a single step. The new hydrogel composite developed in this work has good printability and shape integrity. The combination of a sacrificial material with this new hydrogel composite allows a single step printing of soft and perfusable vasculature-like structures. In the next

chapter, the *in vitro* compatibility of this hydrogel composite will be further investigated by using L929 fibroblasts and human umbilical vein endothelial cells (HUVECs) for proving in potential for vascularized tissue engineering application.

Chapter 5 *In vitro* evaluation of novel bioink and hybrid structure

5.1. Introduction

As mention earlier, the materials used for bioprinting need to be able to support the attachment and survival of cells before and after printing, and it must also be able to degrade appropriately while provide sufficient mechanical strength to create a suitable 3D environment for cells to grow [27, 33]. In order to be used for implants and TE applications, materials' degradation behaviour and cytotoxicity must be evaluated. Therefore, in this chapter, the first experiment is to investigate the degradation behaviour under collagenase, an enzyme which is commonly used for degradation study of GelMA based hydrogel [247, 250]. Next is to investigate the *in vitro* cytotoxicity and cell proliferation on this hydrogel composite by using L929 fibroblasts, which is also commonly used for materials cytotoxicity evaluation [251, 252]. After that, human umbilical vein endothelial cells (HUVECs) are used to evaluate the potential for cell differentiation for vascularization applications. Lastly, to further explore the application of the Plu-GelMA composite, a hybrid structure is fabricated by using Plu-GelMA and cell-laden GelMA that mimic the cross-section of a vascular segment. The details of the experiments will be discussed in each section below.

5.2. Materials and method

5.2.1. Enzymatic degradation test

0.02 wt% Collagenase (Sigma-Aldrich, USA) in PBS was used for enzymatic degradation test of hydrogel composites over 16 days. Hydrogel samples were dried and checked the weight loss every two days. Collagenase solution was changed every 2-3 days over the experimental period.

5.2.2. *In vitro* evaluation of cell viability and cell proliferation

A total of 25 samples of disk-shaped hydrogels (10 mm in diameter and five samples for each concentration) were bioprinted and sterilized by autoclave at 80°C for 15 minutes. Subsequently, samples were plated on a 24-well plate and soaked in cell culture medium for 12 hours before cell culture experiment. L929 mouse fibroblast cells were seeded at a density of 5×10^4 cells/well. Cells were cultivated in low glucose Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) supplemented with 10%(v/v) FBS (PAA, GE Healthcare) and 1%(v/v) antibiotic/antimycotic solution (PAA, GE Healthcare). Culture medium was replaced every 2-3 days and cells were grown at 37 °C in the presence of 5% CO₂. The experiment was sampled at day 1, 3 and 7 for live-dead staining, PrestoBlue® test and SEM fixation. For live-dead staining, the cell culture medium was removed from the sample, followed by washing the samples with PBS for 2-3 times. Live/Dead Cell Double Staining Kit (Sigma-Aldrich, USA) was used to validate cell compatibility of the hydrogels. Solution A (Calcein AM solution) and solution B (propidium iodide solution) were added into each sample at the ratio of 2:1 in PBS solution to form assay solution. 100 µl of assay solution was added into each well and the well was incubated at 37 °C for 15 minutes. Then, cell viability was detected under fluorescence light using a fluorescence microscope with 490 nm excitation for live cells, and at 545 nm excitation only dead cells can be observed. For PrestoBlue® test, the cell culture experiment was stopped by adding PrestoBlue® solution (Invitrogen, Life Technologies, USA) into cell culture medium using a ratio of 1:9 by volume and the well was incubated at 37 °C for 2 hours. Then, the cell number was detected by using a

microplate reader (SPARK 10M, Tecan) with 560 nm excitation and at 590 nm emission. The standard test compared to cell number was shown in Appendix B.

5.2.3. Cell morphology and cell attachment analysis

To investigate the cell morphology on the hydrogel composite, the samples need to be viewed under Scanning electron microscope (SEM). Before that the samples need to undergo SEM fixation as described below. For primary fixation, 2.5%(v/v) glutaraldehyde solution (Sigma-Aldrich, USA) was used. All samples were soaked in 2.5% glutaraldehyde solution for 1 hour at 4 °C. After that, samples were washed with distilled water for several times to remove glutaraldehyde. Next, ethanol was used to dehydrate the cells by a series of concentrations (v/v): 25%, 50%, 70%, 95%, 100% and 100%. Samples were soaked in each ethanol concentration for 10 minutes. After that, samples were washed with distilled water and dried in a desiccator for 1 day. Next, the hydrogel samples were coated with gold at 10 mA for 20 seconds before the SEM examination.

5.2.4. *In vitro* evaluation for cell differentiation and immunostaining

A total of nine samples of disk-shaped hydrogels (10 mm in diameter and three samples for each concentration) were bioprinted and sterilized by autoclave at 80 °C for 15 minutes. Subsequently, samples were plated on a 24-well plate and soaked in cell culture medium for 12 hours before cell culture experiment. HUVECs (Human Umbilical Vein Endothelium primary cells, Lonza) with a passage number of 5 were seeded at a density of 10^5 cells/well. Cells were cultivated in endothelial growth BulletKit (EGM-2, Lonza) supplemented with 1% antibiotic/antimycotic solution (PAA, GE Healthcare). Culture medium was replaced every 2-3 days and cells were grown at 37 °C in the presence of 5% CO₂.

The live-dead staining protocol was same as L929 fibroblast cells. For immunostaining, the cell differentiation of HUVECs was investigated at day 10 by using CD31 and von Willebrand Factor (VWF) expression. The samples were rinsed in DPBS a few times and fixed in 4% formaldehyde solution (Sigma-Aldrich, USA) in Dulbecco's Phosphate Buffered Saline (DPBS) (Hyclone, GE life science) for 30 minutes. After that the samples were soaked in blocking solution (5%wt BSA, 0.5%wt Tween in DPBS) for 2 hours at room temperature. Subsequently, the cell membranes were permeabilized in 0.25%(v/v) Triton X-100 (Bio-Rad, USA) in blocking solution for 20 minutes and washed with DPBS for three times. The samples were soaked in the primary antibody staining with 1/100 dilution of mouse monoclonal anti-CD31 antibody (Life technologies, Thermo fisher) and 3 µg/ml of VWF mouse monoclonal antibody (Life technologies, Thermo fisher) in DPBS overnight at 4 °C. The samples were washed with blocking solution three times with 5 minutes intervals between the washing steps. After primary antibody staining, the samples were incubated in 1/1000 dilution of Alexa Fluor-488 conjugated goat antimouse (Life technologies, Thermo fisher) and 1/500 dilution of Alexa Fluor-555 conjugated goat antimouse secondary antibodies (Life technologies, Thermo fisher) in DPBS for 2.5 hours at ambient condition (Alexa Fluor 488 was paired with VWF and Alexa Fluor 555 was paired with CD-31). Subsequently, the samples were washed in blocking solution three times with 15 minutes intervals between the washing steps, followed by 5 µl/ml of DAPI (NucBlue®, Thermo Fisher) staining for 20 min. After rinsing, fluorescent images were taken by using florescent microscope (Axio Vert.A1, Carl Zeiss, Germany). For SEM fixation of HUVECs, the

samples were collected at day 1 and day 7 and followed the protocol as mentioned above.

5.2.5. Hybrid structure fabrication and cell testing

The hybrid structure was designed based on the anatomical structure of blood vessel. The core structure was printed by using Plu-GelMA 2:1 hydrogel composite and the internal part was cell-laden GelMA hydrogel (5 wt%). HUVECs was loaded inside GelMA at a density of 3×10^6 cells/ml. All the printing conditions were the same as described in the printability section of Chapter 4. After printing, the structures underwent UV curing and were cultured in EGM-2 medium for 7 days. The live-dead staining protocol was the same as described earlier.

5.2.6. Cross-sectioning and Hematoxylin and Eosin (H&E) staining

The samples were fixed with formaldehyde (10%) overnight followed by dehydrated with ethanol by a series of concentrations (v/v): 70%, 80%, 95% (2 times), and 100% (3 times). Samples were soaked in each ethanol concentration for 45 minutes. Later, samples were soaked in xylene for 3 times with 45 minutes interval between each time. Subsequently, the samples were embedded in paraffin and 7 μ m sections were cut by using a microtome (Leica, Germany). The sections were immersed in water bath at 37°C and mounted on Polysine® adhesive coated glass slides for better adhesion. The slides were left to dry overnight at 37°C. The sections were rehydrated in xylene, then a decreasing series of alcohol concentrations: 100%, 95% and 70%, followed by immersing in water. Conventional haematoxylin and eosin (H&E, Sigma, USA) staining was performed. Slides were examined under light microscopy (Carl Zeiss, Germany).

5.2.7. Statistical analysis

The statistical significance was determined by Student t-test study for two groups of data or analysis of variance. P-values were presented as statistically significant and highly significant as * $P < 0.05$ (at 95% level of confidence), ** $P < 0.05$ is for these significantly different from the rest.

5.3. Results and discussion

5.3.1. Enzymatic degradation test

By using 0.02 wt% collagenase, the enzymatic degradation of Plu-GelMA hydrogel composite was studied. As expected, the greater GelMA content led to a slower degradation rate (as shown in Figure 5.1) due to more chemically crosslinked bonds [247]. Similar to most of GelMA hydrogel base, the mass of the hydrogel composite significantly dropped from day 1 to day 6 and the drop became slower afterward [253]. Plu-GelMA hydrogels ratio 2:1 and 1.5:1 completely degraded after 14 days. The rest stayed until day 16 before fully degraded.

Figure 5.1. Enzymatic degradation study of Plu-GelMA hydrogel composite at different ratio over 16 days in 0.02 wt% collagenase (all groups $n = 3$, error bars represent standard deviation).

5.3.2. *In vitro* evaluation of cell viability (cytotoxicity) and cell proliferation

L929 fibroblast cells are commonly used for preliminary biocompatibility and toxicity test [251, 254, 255]. As shown in Figure 5.2a, all Plu-GelMA of different mass ratios were biocompatible and supported cell proliferation, among which Plu-GelMA 2:1 achieved the highest number of cells at day 7. This can be further evidenced by live/dead staining (Figure 5.2b) of L929 fibroblasts on Plu-GelMA 2:1.

From *in vitro* evaluation, L929 cells were alive and proliferated over 7 days on all composites. The Plu-GelMA 2:1 achieved the highest cell number compared to other ratios, perhaps due to higher swelling ratio and larger pores in microstructure. The larger pores can lead to more surface area and better medium transportation through the entire structure. The L929 cells were able to attach onto the hydrogel composites and able to pack together when the cell number increased. This proved that the Plu-GelMA 2:1 hydrogel composite provided a good platform for cell attachment and proliferation.

Figure 5.2. L929 *in vitro* cell viability and cell proliferation test: **a)** PrestoBlue cell proliferation test of Plu-GelMA hydrogel from day 1 to day 7 ($n = 3$, statistical significance determined by pairwise t-test where $*P < 0.05$, $** P < 0.01$ is for significantly different from the rest of the data set) and **b)** Live/dead staining for cell viability test of L929 cells on Plu-GelMA 2:1, the images were observed under microscope at 5x magnification (scale bars, 200 μm).

5.3.3. Cell Morphology investigation on hydrogel composite

As shown in Figure 5.3, for Plu-GelMA 1:2, L929 cells seemed to spread a bit more on the surface at both day 1 and day 7 as compared to Plu-GelMA 2:1. However, at day 7, some of the cells were getting rounder similar to that on Plu-GelMA 2:1 due to the effect of GelMA in the composite. Normally, protein based

hydrogel or protein coating leads to more spreading type of cell morphology [73, 251, 256].

Figure 5.3. SEM fixation at 500x magnification of L929 cells on 1:2 Plu-GelMA hydrogel composite.

On the other hand, for ratio 2:1 as shown in Figure 5.4, the L929 cells were able to attach onto the hydrogel composites, proliferate and contract the hydrogel, resulting in the highest number of cells on the surface as shown in the Prestoblue results at day 7 (Figure 5.2a). For Plu-GelMA 2:1, even though cell morphology was a bit rounder at day 1, they spread more evenly as compared to ratio 1:2 and were able to contract the hydrogel to a larger degree due to larger pore structure. This result shows that ratio 2:1 is better for cell proliferation.

Figure 5.4. SEM fixation at 500x magnification of L929 cells on 2:1 Plu-GelMA hydrogel composite.

5.3.4. *In vitro* evaluation for cell differentiation and immunostaining

HUVECs are commonly used for angiogenesis study and vascular tissue engineering [257, 258]. In this research, they were used to further evaluate Plu-GelMA for supporting cell differentiation. After 10 days of culture, the live/dead staining and immunofluorescence results (Figure 5.5a) show that HUVECs were fused and alive until day 10 and endothelium cell markers CD31 and VWF were expressed. SEM images (Figure 5.5b and 5.5c) show that HUVECs were able to attach and spread on the Plu-GelMA 2:1 surface, and at day 7, they fused and formed layers covering the hydrogel surface.

Figure 5.5. HUVECs *in vitro* cell evaluation: **a)** Live/dead staining and immunofluorescent of HUVECs on Plu-GelMA 2:1 at day 10, the images were observed under microscope at 40x magnification (scale bars, 20 μ m), **b)** SEM fixation at 1000x magnification of HUVECs on Plu-GelMA 2:1 (day 1) with false colour to present cells attachment and **c)** HUVECs on Plu-GelMA 2:1 at day 7.

The results of HUVECs study prove that the Plu-GelMA 2:1 hydrogel composite supported cell differentiation, evidenced by the expression of two markers of endothelium cells - CD31 and VWF. CD31 shows the formation and fusing of HUVECs [258] which may lead to the angiogenesis and vascular branching [243] if the cell culture is continued. The presence of VWF shows the efficiency of vascularization [259]. Lastly, as shown in Figure 5.5b, primary HUVECs could also attach and spread on the composite surface. At day 7, when the number of

cells was sufficient, the HUVECs fused and covered the hydrogel composite surface with their extracellular matrices, which confirmed that this hydrogel composite was able to support attachment for different types of cells.

5.3.5. Hybrid structure fabrication and *in vitro* evaluation

The hybrid structure was inspired by the anatomy of the blood vessels as shown in Figure 5.6a. In this part, the HUVECs were embedded into GelMA instead of Plu-GelMA because of opacity and Plu-GelMA was used as the core structure to create patterned due to its printability.

Figure 5.6. Vascular inspired hybrid structure fabrication: **a)** Artery, vein and capillary anatomy inspired (Copyright © 2013 Pearson Education, Inc.) [260], CAD design and actual printed parts and **b)** Live/dead staining of HUVEC cells in hybrid structure at day 3, the images were observed under microscope at 10x magnification (scale bars, 100 μm).

The bioprinted results showed that this technique provided good resolution and the multilayer design could be varied from few layers that simply mimic a capillary to many layers that are structurally similar to arteries and veins. The live-dead staining was performed at day 3 of cell culture and from Figure 5.6b, it

showed that majority of HUVEC cells were alive and aligned following the circumferences of the hybrid structure.

5.3.6. H&E of L929 seeded samples and the cross-section of hybrid structure

The H&E test is to stain nuclei of the cells and the protein for fixed tissue or *ex vivo* tissue samples [261, 262]. However, nowadays it has been used for investigating cell-material interaction and dual-material interaction [263, 264]. As shown in Figure 5.7 below, the L929 seeded samples on 2:1 Plu-GelMA hydrogel composite represented that cells were able to integrate and stay in the hydrogel as the nucleus of L929 cells can be seen in the sample.

Figure 5.7. The H&E image of L929 on the Plu-GelMA 2:1 hydrogel composite at 5x (top) and 20x (bottom), the red arrows pointed the cell nucleus.

For hybrid sample without cells, the materials integration and interaction were investigated. As shown in Figures 5.7 and 5.8, Plu-GelMA cracked under the dehydrated condition and appeared darker in colour as compared to pure GelMA largely due to lesser amount of protein produced by cells. The eosin Y stains protein and when there are more protein contents, it gives rise to pink or red colour [261]. Lastly, there was some interaction at the border between Plu-GelMA and GelMA. Some of the GelMA were able to mix with Plu-GelMA due to their difference in concentration, which led to a difference in osmotic pressure. However, the interaction was quite minor since most of the GelMA still stayed in compact layer as shown by the red arrow in Figure 5.8.

Figure 5.8. The H&E image without cells of hybrid structure at 5x, the red arrow presented GelMA region while the yellow arrow presented Plu-GelMA.

5.4. Summary

From *in vitro* evaluation, it has been proved that the Plu-GelMA composite had low toxicity since most of the cells were alive and proliferated until day 7. The 2:1 ratio Plu-GelMA showed the best cell proliferation due to its porous structure. In addition, the 2:1 Plu-GelMA hydrogel composite provided a good platform for cell differentiation as shown in the HUVECs experiment. The HUVECs test proved that the Plu-GelMA hydrogel composite supported cell differentiation and

the two markers of endothelium cells, CD31 and VWF, were observed. The presence of the endothelial cell markers demonstrated the efficiency of vascularization, which could be a potential of Plu-GelMA composite for vascularization applications. Lastly, the hybrid structure - comprising HUVECs embedded in 5%wt GelMA and Plu-GelMA as a core - proved to support cell alignment with good viability. The design of the hybrid structure was inspired by the anatomy of the blood vessel. Based on the *in vitro* evaluation with HUVECs, it could potentially be used for vascular implants, drug testing or other related vascular applications. In the next chapter, another possible strategy for vascularization which is cell sheet transfer on a solvent-free membrane will be discussed.

Chapter 6 Fabrication, characterization and surface modification of biodegradable membrane

As mentioned earlier in the first chapter, the high cell density approach is an alternative for solving TE problems especially for endothelial cells in order to promote dense vessel structures [265]. Unlike cell sheet technology, membrane also can be served as a cell carrier due to large surface area and porous structure. Moreover, the mechanical strength of membrane makes it more suitable for handling and transfer compare to cell sheet. Thus, the membrane has a potential to be used as a transfer platform for a large number of cells. In order to create high cell density on to the membrane's surface, the membrane must be toxic free and suitable for cell proliferation and cell attachment.

Therefore, in the first part of this chapter, surface suspension melt technique, a novel method for fabricating a solvent-free, micro-porous, biodegradable membrane, is presented. This method uses water to fabricate membranes, eliminating the use of organic solvent. The water serves as a substrate and platform for a thin layer of polymer particles such that this mixture becomes a surface suspension system, i.e. the particles are dispersed throughout the surface of water, not the bulk of water. Conventionally, there are three types of mixtures based on particle size: solutions, colloids and suspensions. Suspensions normally refer to mixtures of liquids and coarse solid particles (size > 1 μm) in which particles can be seen on a macroscopic scale. Eventually after some period of time suspension particles will sink. However, in this experiment, the evenly dispersed particles always stay on the water surface without sinking down, making the mixture a "surface suspension system". The second part of this

chapter focuses on characterization of biodegradable membranes. The last part is on the membrane surface modification by using plasma and protein grafting to improve hydrophilicity and cell adhesion.

6.1 Surface suspension melt membrane fabrication

6.1.1. Materials and method

Polycaprolactone (PCL) powder (CAPA® 6501, Molecular weight: 50 kDa, average particle size 100 μm) was purchased from Solvay Interlox, Warrington, UK. The material density of polycaprolactone is 1.1 g/cm^3 . The melting point is around 60 $^{\circ}\text{C}$. Another size of PCL powder is 500 μm PCL powder which was purchased from Perstorp (Malmö, Sweden). The CAPA® PCL powder was sieved with a No. 140 sieve to confirm its 100 μm powder size. For 500 μm powders, the particle size can be confirmed by sieving with a No. 35 sieve as shown in the 200x magnification SEM image in Figure 6.1a and 6.1b.

Figure 6.1. a) SEM image of 100 μm dry PCL powder at 200x, b) SEM image of 500 μm dry PCL powder at 200x and c) Schematic illustration of a micro-porous membrane fabrication starting from a micro-particles powder layer forming a surface suspension mixture.

As shown in Figure 6.1c, to facilitate the preparation of a layer of micro-particles, a liquid substrate was used as a platform for the powder and to allow an even distribution of powder particles as reported in our previous studies [266, 267]. Briefly, 30 ml of deionized water was prepared and poured into a round glass dish (diameter: 10 cm) at room temperature. 100 μm PCL powder (0.1 g) and 500 μm PCL powder (0.7 g) was weighed and slowly dispensed onto the water surface to form a surface suspension system. The glass dish was then gently shaken circularly to allow the microparticles to distribute evenly on the water surface. A rubber bulb was used to gently blow off and spread the excess powder. Subsequently, the glass dish was lightly shaken for another 10 seconds to allow the particles to distribute evenly. Next, for 100 μm powder, the glass dish was placed on the bottom level of a laboratory oven (BINDER, Inc., Bohemia, NY, USA) and the temperature was set at 80 °C. After 20 minutes, the glass dish was taken out and cooled at room temperature until a solid white membrane appeared. On the other hand, for 500 μm powder, the glass dish was put onto a laboratory heating plate (Thermolyne Cimarec® 1, Thermo Scientific, Waltham, MA USA) and the heating level was set at level 2 through all the experiments. After 15 min, the heater was turned off and the glass dish was removed from the heater. Finally, the glass dish was allowed to cool down naturally at room temperature. After about 5 minutes, a solid white membrane appeared on the top of the water surface. A total of 20 membranes (ten membranes for each particle size) were fabricated.

6.1.2. Results and discussion

6.1.2.1. Dispersion of a layer of PCL particles

Surface suspension melt technique is a novel method of fabricating PCL micro-porous membranes. It involves three steps: preparation of a layer of particles on

a water surface to form a suspension mixture, heating this mixture to form a melted layer, and cooling to form a solid membrane layer. Figure 6.2 shows a layer of PCL particles during preparation. Dispensing was completed by gently tapping the weighing boat. The glass dish was gently shaken with repeated circular motions. Distribution was aided by blowing with a repeatedly squeezed rubber bulb. Normally, suspension mixtures lead to the sedimentation of particles at the bottom of the glass Petri dish. However, PCL powder particles are small, light and highly hydrophobic, so 0.1 g of 100 μm PCL powder can float on the water surface because of water surface tension. The powders used in this experiment were not enough to agglomerate and sink. In the case of 500 μm powders, they behaved the same way as 100 μm powders but required more powder (in terms of weight) to cover the same area.

Figure 6.2. Preparation of a layer of PCL particles on the water surface and melting state layer formation in a 10 cm diameter glass dish.

6.1.2.2. Fabrication of a membrane from a layer of PCL particles

The concept that a layer of micro-particles can be directly formed into a layer of membrane is demonstrated in Figure 6.1 and 6.2. Both figures indicate that the

membrane can be formed by the powder melting on top of water surface with the use of heat. In surface suspension melt, water serves as a heat medium and as a platform for PCL particles in both the solid and melted state. Water allows the particles to stay on top by the effect of surface tension between the hydrophobic polymer and water. Figure 6.3a shows a layer of dispersed particles spread on the water surface in the form of a surface suspension mixture. Figure 6.3c is a zoomed-in view that shows a microscopic observation of a layer of particles resting only on the water surface. The fabricated PCL membrane can be found in Figure 6.3b. It was semi-transparent and soft, and had a similar size as the glass dish. The zoom-in view shows the membrane is solid and micro-porous, as indicated by the microscopic view in Figure 6.3d. The initial powder layer consists of irregularly shaped particles but the membrane is a solid piece with pores.

Figure 6.3. a) Macroscopic of the powder dispersion on the water surface, b) Macroscopic view of membrane made from 100 µm powder, c) Microscopic image of the powder dispersion under a light microscope with 10x magnification, d) Microscopic image of membrane made from 100 µm powder surface (SEM at 100x), e) Macroscopic image of membrane made from 500 µm powder and f) Microscopic image of membrane made from 500 µm powder surface (SEM at 100x).

It should be noted that during the powder blowing process, some powder was blown out of the glass dish or firmly adhered onto the glass wall. Also, the temperature of 80 °C was chosen due to the fact the melting point of polycaprolactone is around 60 °C and the boiling point of water is 100 °C. Figure 6.2 demonstrates that when the PCL powder layer was completely melted, it changed from an opaque solid to a transparent liquid. The fabrication temperature must be able to melt PCL particles whilst not boiling water. The fabrication time of 20 minutes was chosen as only a ring of particles near the wall of the glass dish melted and the entire layer of particles remained white and opaque at 10 minutes. At 30 minutes, the membrane was found to shrink in size due to melt surface tension. PCL is a hot adhesive material. When PCL is melted, it is able to adhere to glass. If the edge of the melted layer firmly attaches to the glass wall, the melted layer will not shrink as the attachment resists the contraction. However, if some part of the edge happens to detach from the glass wall due to insufficient coverage of powder and the resulting poor attachment, a hole may form near the edge of the membrane. A smaller but thicker membrane can be formed after cooling as a result of the detachment. To prevent these problems, the blowing step in the preparation of a layer of particles is critical. Lastly, the glass border of the Petri dish not only limits the spread of the powder but also facilitates the circumferential attachment of a layer melt. These methods actually can be repeated and used for different size of particles. However, this method is manual and depends a lot on user experience and the way powders are dispensed on top of the water still needs further improvement for better control.

The solvent-free surface suspension melt technique has a number of advantages. Firstly, the process does not involve any organic solvent or additives, which is

particularly important for tissue engineering, because toxic chemical residues are likely to alter the behaviour of cells or cause cell death. In order to ensure that there is no organic solvent residue left, post-processing is required to remove all the residual solvent out of membrane. These post-processing steps can take around 1–2 days [207] so by using this method, post-processing is negligible. Secondly, it is fast. The method only requires a common laboratory oven and the membrane can be formed within less than 30 minutes. Thirdly, this method effectively utilizes the spaces among micro-particles, which eventually leads to the pores on the membrane, enabling the fabrication of a micro-porous membrane without the need for surface modification post-processing. Even though mechanical drilling and laser drilling can be used to confirm the uniformity of pore size and pore position on films or membranes, mechanical drilling can lead to problems of tool breakage and force-induced cutting. On the other hand, for laser drilling, many parameters have to be controlled carefully to avoid an issue of heat-affected zone (HAZ) that may affect materials' properties [268, 269]. Finally, the substrate in this method is a liquid. The fluidity of liquids allows convenient detachment of a flat membrane, avoiding bent or ruffled shapes. Therefore, this method can overcome the challenge of biodegradable membrane fabrication purifying post-processing because it can generate micro-porous membranes in a fast and straightforward manner without any use of organic solvent that will be harmful for cells and tissues. However, some limitations should also be addressed. The materials have to be in the form of a fine powder, as coarse particles affect the texture of the membrane. Additionally, the powder has to be insoluble in the liquid substrate. Otherwise, the particles will dissolve

in the liquid. Furthermore, the melting point of the powder has to be lower than the boiling point of the liquid substrate.

6.2. Characterization of membranes

6.2.1. Materials and method

6.2.1.1. Membrane morphology

Light microscopy (CKX41, Olympus, Tokyo, Japan) was used to examine the morphology of particles on the water surface and the failure morphology was examined by scanning electron microscopy (SEM, JSM-5600LV, Jeol, Tokyo, Japan). The PCL membrane was cut into 10 mm × 10 mm square pieces and coated with gold at 10 mA for 20 seconds before the SEM examination.

6.2.1.2. Thickness measurement

A micrometer (Coolant Proof Micrometer Series 293, accuracy: ± 0.00127 mm, Mitutoyo, Tokyo, Japan) was used to measure the thickness of six PCL membranes for each powder particle size. For each membrane, ten measurements were taken at points selected randomly.

6.2.1.3. Pore size measurement

Three membranes for each size of powder particle were used in this characterization test. Each membrane was cut into five rectangular samples (25 mm × 15 mm) using a surgical blade from five parts (top, bottom, left, right and center) of the same membrane and sputter coated with gold at 10 mA for 20 seconds before SEM measurement (JEOL JSM-5600LV). All SEM images were taken at a fixed magnification of 100x. Pore size was measured using the software SmileView (Version 2.05).

6.2.1.4. Surface roughness measurement

The surface roughness, R_a , of the PCL membranes was measured by using the Confocal Imaging Profiler (PL μ , SENSOFAR, Terrassa, Spain). For each size of

powder particle, six membranes were investigated with four readings taken at four different positions.

6.2.1.5. Mechanical tests

Mechanical properties of the PCL membranes were tested at room temperature using a Model 5547 Microtester (Instron, Norwood, MA, USA). A total of 10 samples (10 mm × 15 mm) were cut from two PCL membranes for each powder particle size and prepared by sandwiching the two extreme ends between two pieces of cardboard. The cardboard ends were then mounted onto the clamps of the Instron Microtester. Loads of 50 N and strain rate of 10 mm/min were applied.

6.2.1.6. Crystal structure study

Membranes were embedded in resin by using Leica HistoResin kit (Leica, Germany). Then the embedded samples were cut by using microtome (RM 2155, Leica, Germany) with the thickness of 10 µm per slice. The sliced samples were put under the polarized microscope to investigate the crystal structure of membranes.

6.2.1.7. *In vitro* evaluation of membranes for cell compatibility

Cell culture

For cell culture tests, only membranes made from 100 µm PCL powder were used. C2C12 murine myoblasts were plated on the PCL membrane coated wells of a 24-well plate. PCL membranes were held in position with circular hollow metal rings to prevent them from floating. Before cell seeding, PCL membranes were sterilized using 70% ethanol for 1 hour and washed with phosphate buffer saline (PBS) several times. Cells were seeded at the density of 2×10^5 cells/well. Cells were cultivated in low glucose Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% FBS (PAA,

Pasching, Austria) and 1% antibiotic/antimycotic solution (PAA). Culture medium was replaced after every 2–3 days and cells were grown at 37 °C in the presence of 5% CO₂. For cell detachment purpose, 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) solution was utilized.

Immunocytochemistry and microscopy

Cells were fixed for 10 minutes in 4% paraformaldehyde and permeabilized for 15 minutes in 0.1% Triton X-100 at room temperature. Having washed the cells twice with PBS, for blocking purpose, 5% bovine serum albumin was used for 1 hour at room temperature. Then, cells were incubated with primary antibody against mouse monoclonal anti heavy chain cardiac myosin (1:400, Abcam, Cambridge, UK) overnight at 4 °C, followed by washing with PBS for three times. After 1 hour incubation with Alexa Fluor 488 goat anti mouse IgG (1:400, Molecular Probes, Eugene, OR, USA), cells were rinsed with PBS several times. Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, 1:400, Chemicon, Temecula, CA, USA). To validate the performance of heavy chain cardiac myosin primary antibody, C2C12 myoblasts were cultured in 2% horse serum to induce myogenic differentiation and stained for myosin heavy chain. Images were captured using an Eclipse 80i upright microscope (Nikon, Tokyo, Japan) with 10x objective lens and image analysis was done by using ImageJ 1.44f software.

Cell proliferation assay

A PicoGreen assay was implemented to check the proliferation rate of C2C12 myoblasts seeded on the PCL membrane. Initially, the PCL membranes covered with cells were rinsed carefully three times in PBS and immersed in 0.5% Triton X-100 solution to permeabilize cells with gentle pipetting for 30 min. According

to the manufacturer's instructions, PicoGreen working solution was prepared by diluting concentrated PicoGreen reagent in TE buffer (1:200). Then, equal quantities of cell lysate and PicoGreen working solution were mixed together in the wells of a 96-well plate and incubated for 5 minutes at room temperature. Finally, fluorescence emission readings were taken using an Infinite200® microplate reader (Tecan Inc., Mannendorf, Switzerland) at 520 nm under an excitation wavelength of 480 nm. Cell proliferation rate was measured at different time intervals ranging from day 1 after cell seeding to day 6.

6.2.2 Results and discussion

6.2.2.1. Membrane surface characteristics and mechanical strength

The membrane surface characteristics and mechanical properties, including thickness, roughness, stiffness and ultimate tensile load are presented in Table 6.1.

Table 6.1. Membrane characteristics and mechanical properties.

Parameter	Membrane Properties	
	From 100 μm Powder	From 500 μm Powder
Thickness	$27.3 \pm 2.8 \mu\text{m}$	$134.9 \pm 3.6 \mu\text{m}$
Roughness (R_a)	$3.4 \pm 2.9 \mu\text{m}$	$5.5 \pm 3.0 \mu\text{m}$
Stiffness	$2.40 \pm 0.40 \text{ N/mm}$	$0.15 \pm 0.02 \text{ N/mm}$
Ultimate tensile load	$1.6 \pm 0.3 \text{ N}$	$10.1 \pm 2.5 \text{ N}$

As shown in Table 6.1, the thickness of membrane is much smaller than the size of the PCL particles used. Initially, particles should form a layer of a thickness of about 100 μm or 500 μm as well as a network of voids among these particles due to their irregular shapes. When particles are melted on the water surface, they become round and spread, and fill in their adjacent spaces. The overall thickness of the layer is thus reduced as a result of material redistribution. As expected, Table 6.1 also shows that a bigger particle size leads to a thicker membrane. For mechanical properties, the thicker membrane resulted in a higher ultimate load,

but lower stiffness. Figure 6.4 is a SEM image of the cross-section of a membrane, which shows the membranethickness. The measured results of the membrane roughness may be used to explain the perception of a fine texture of the membrane. It is the small particles used in the fabrication that resulted in an even and smooth membrane surface. Moreover, the consistency in particle sizes and natural flatness of the water surface may also contribute to the overall evenness.

Figure 6.4. The cross-section of a PCL membrane made from 100 µm powder.

Figure 6.5 shows the structure of a stretched membrane. It can be seen that the pores were the place that cracks started to occur. Small cracks adjacent to each other converge into a long crack perpendicular to the loading. The thinning process usually started at the periphery of pores and regions with less or no pores decreasing in size and becoming connected by multiple thinned strips as shown in Figure 6.5b.

Figure 6.5. Failure mode **a)** Near the sample holding grip and **b)** In the middle of a sample.

6.2.2.2. Distribution of pore Size

As shown in Figures 6.3d and 6.3f, black spots represent the membrane pores which show that the pores' size varied. Figure 6.6 and Table 6.2 summarise the pore size distribution of three membranes. The pore size ranged from a few micrometers to nearly one hundred micrometers, and the average pore sizes for the three membranes were $16.4 \pm 8.8 \mu\text{m}$ for membrane made from $100 \mu\text{m}$ powder and $95.4 \pm 42.5 \mu\text{m}$ for membrane made from $500 \mu\text{m}$ powder, respectively. By comparing the pore size from membrane made from $100 \mu\text{m}$ and $500 \mu\text{m}$ powder in Table 6.2, the results show that a larger particle size leads to more variations in pore size. Smaller powder leads to more consistency in membrane pore size. However, the pore distribution trend was the same for the two different membranes as illustrated in Figure 6.6.

Figure 6.6. Pore size distribution of **a)** membranes made from 100 μm powder and **b)** membranes made from 500 μm powder.

Table 6.2. Distribution of pore size of three membranes made from 100 μm and 500 μm powder.

Powder size	Sample	1	2	3
100 μm	Number of pores measured	949	885	1000
	Average (μm)	16.2 ± 9.2	16.7 ± 10.9	16.2 ± 6.3
	Max size (μm)	95	80	46.1
	Min size (μm)	2	3	6
500 μm	Number of pores measured	996	1016	1000
	Average (μm)	151.7 ± 70.7	61.2 ± 30.5	73.3 ± 26.2
	Max size (μm)	350	288	347
	Min size (μm)	14	11	14

6.2.2.3. Crystal structure study

After slicing membrane by using microtome in order to get 10 μm layer, the sliced samples were investigated for crystal structure by using polarized microscope. In Figure 6.7, the results illustrate that by comparing to solvent

casted film made from PCL powder and chloroform, crystal structure of solvent-free membrane is relatively smaller. Due to different formation phenomena, the results of crystal structure are significantly different. From the work of Vincent *et al.*, the PCL crystal growth depends on crystalline temperature, and at a lower temperature, lamellar crystal structure is larger compared to higher crystalline temperature [270]. Solvent cast film was formed at room temperature (25 °C) with the endothermic reaction of solvent evaporation, solvent-free membrane was heated up and crystalline temperature might shift to be higher than room temperature. Moreover, for the solvent-free fabrication, there is a sudden change in temperature of more than 50 °C from 80 °C (fabrication temperature in an oven) to room temperature. This sudden change leads to less time for the polymer chain to align and form crystal. Therefore, the crystal structure of the solvent-free membrane is smaller than the crystal of solvent cast film.

Figure 6.7. Crystal structure from polarized microscope **a)** PCL membrane made 100 µm powder at 60x, **b)** PCL membrane made 500 µm powder at 60x and **c)** Solvent casted film at 60x. Scale bar represent 20 µm long.

6.2.2.4. *In vitro* cell compatibility study

In order to investigate the cell proliferation rate, C2C12 myoblasts grown on biocompatible PCL membranes were subjected to a PicoGreen DNA quantification assay. Figure 6.8a shows the cell DNA concentration per PCL membrane at different time intervals. During the DNA quantification assay, the DNA concentration was found to be around 1000 ng/ml, 1 day after cell seeding. Remarkably, at day 2, there was a four-fold increase in the cell DNA concentration (4000 ng/ml). It has already been reported that the C2C12 doubling time is approximately 12 hours [271, 272]. Hence, the observed fourfold increase in DNA concentration after 24 hours is feasible. At day 3, a sudden decline in DNA concentration was observed. Moreover, the DNA concentration gradually and continuously decreased till day 6 and finally it fell below 1000 ng/ml. The cell proliferation results showed that at day 2, C2C12 reached 100% confluence. This contact inhibition of C2C12 growth coupled with withdrawal from the proliferation cycle as myoblasts fused to form myotubes, causing a down-turn in cell population as seen from day 3 onwards. Nevertheless, the results from the first two days imply that the PCL membrane provides good support for cell attachment and proliferation.

MHC immunostaining of cells grown over PCL membranes after 4 days of culture showed distinct myotube formation (Figure 6.8b). Previous reports have elucidated the systematized skeletal myogenesis process and revealed the connection between the different steps that control the fusion of mononucleated myoblasts into terminally differentiated multinucleated myotubes [273, 274]. Differentiated myotube formation over PCL membrane specifically shows the biocompatibility of PCL membrane for muscle tissue engineering. The positive

control (Figure 6.8c) shows the myotube formation in C2C12 cells induced by 2% horse serum. These cells grew in the tissue culture plastic dish for 4 days before performing the immunostaining. Finally, the results of *in vitro* of C2C12 myoblast cell culture indicated that the membrane was able to support cell growth and differentiation.

Figure 6.8. a) DNA concentration of C2C12 myoblasts found at different time intervals in PicoGreen assay, b) MHC staining (green) displayed myotube formation on PCL membrane after 4 days of cell seeding and c) Positive control demonstrates induction of myotube formation in C2C12 myoblasts grown in DMEM containing 2% horse serum.

6.3. Membrane surface modification

PCL has good biocompatibility, but the initial cell adhesion is low due to high hydrophobicity. To overcome this problem, solvent free PCL membrane needs to undergo surface modification with protein such as gelatin and collagen. In this

research, PCL membrane is treated with plasma followed by gelatin and collagen immobilisation.

6.3.1. Materials and method

6.3.1.1. 2³ factorial experiment for gas plasma study

In this experiment, three factors were considered at 2 levels, high (+) and low (-). The factors are: type of gas plasma (A): argon (+) or oxygen (-), flow rate (B): 200 and 100 sccm and treatment time (C): 180 and 120 seconds. Eight sets (2³) of experiment, each with five specimens of PCL membranes, were carried out subjected to the above conditions using plasma reactor (MARCH PX-500), as shown in Table 6.3 below. At 100% flow rate of 250 sccm and 500 sccm, the conversion factor of argon and oxygen are 1.44 and 0.994 respectively. Percentage of gas desired was calculated as shown in Appendix C. The rest of parameters which are Radio frequency (RF), power and displacement were kept constant at 13.56 MHz, 100 W and the PCL membranes were placed at the centre position.

Table 6.3. Conditions for 2³ plasma treatment study membrane surface modification.

Run	A	B	C	Type of gas	Flow rate (sccm)	Argon content (%)	Oxygen Content (%)	Treatment time (s)
1	-	-	-	O ₂	100	-	20	120
2	+	-	-	Ar	100	28	-	120
3	-	+	-	O ₂	200	-	40	120
4	+	+	-	Ar	200	56	-	120
5	-	-	+	O ₂	100	-	20	180
6	+	-	+	Ar	100	28	-	180
7	-	+	+	O ₂	200	-	40	180
8	+	+	+	Ar	200	56	-	180

6.3.1.2. Gelatin coating

In this experiment, gelatin was used to reduce PCL hydrophobicity and to increase cell attachment. The procedure of preparing gelatin (G2500, Sigma, MO,

USA) solution is as follows: five grams of gelatin powder was mixed with 50 ml of deionized water to obtain final concentration of 2.5% w/v. PCL membranes firstly underwent argon plasma treatment (Ar: 28% at 100sccm, 120s) and later were immersed in the 2.5% w/v gelatin solution at 37 °C for 2 dys. After immersing, the specimens were dried in desiccator for 1 day, and rinsed in phosphate buffered saline (PBS, Sigma, USA) solution to remove excess gelatin. Thereafter, the specimens were left in a desiccator to dry for another day. Gelatin coated scaffolds were ready for further surface modification and characterization.

6.3.1.3. Collagen immobilisation

This method was adapted from the work of Cheng *et al.* [204] as shown in Figure 6.9. Briefly, PCL membranes first underwent argon plasma treatment (optimum condition: Ar: 28% at 100 sccm, 120 s) and later all samples were immersed in the solution containing 0.11 g/ml Acrylic acid, (AAc, Sigma, USA) and Vitamin B (Riboflavin, Sigma, USA). Subsequently, samples were washed with PBS to remove non-reacted AAc.

Figure 6.9. Schematic of collagen immobilisation process.

PCL-AAc grafting membranes were soaked in water soluble N-(3-Dimethylaminopropyl)-N'-ethyl carbodiimide hydrochloride, (E7750, Sigma) for 1 hour at 4 °C. Then, samples were treated in 0.5 g/ml collagen type I solution (human, C7624, Sigma) for 5 hours at 4 °C. Finally, the samples were immersed in PBS for 1 hour to remove excess collagen and stored in refrigerator for further experiment.

6.3.1.4. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

Membranes were characterized in ATR mode by using Thermo Scientific Nicolet™ 6700 FT-IR spectrometer (Cambridge, UK) which was equipped with OMNIC software. Samples were mounted on to the orbit sampler. The spectra's results were demonstrated in the range of 500 – 4000 cm^{-1} with a resolution of 4 cm^{-1} under absorbance mode.

6.3.1.5. Differential scanning calorimetry (DSC)

DSC was performed on the different types of surface modified PCL membranes such as plasma treatment, gelatin coating and collagen immobilisation. DSC results are able to demonstrate changes in thermal transitions of the membranes before and after the modification. The DSC (Q200, TA Instruments, USA) characterization was performed with working condition of temperature ranges from 20 to 80 °C with the ramp rate of 20 °C per minute.

6.3.1.6. Water contact angle

Water contact angle characterization was carried out using Attension (Finland) Theta Optical tensiometers with the droplet size of 5 μl . Five replicates for each specimen were tested to determine the differences in degree of hydrophobicity of

different modified PCL surface. The Attension software was attached to the machine and used for contact angle analysis.

6.3.1.7. *In vitro* evaluation of surface modified membrane using L929 fibroblasts

Nine samples of 10 mm x 10 mm membranes which included non-treated, gelatin coating and collagen immobilisation (three for each) were sterilized by soaking with 70% ethanol and put under UV light for 1 days. Subsequently, samples were soaked in cell culture medium for 12 hours before cell culture experiment. Samples were plated on a PCL membrane coated wells of a 24-well plate. PCL membranes were held in position with circular hollow metal rings to prevent them from floating. L929 mouse fibroblast cells were seeded at the density of 3×10^5 cells/well. Cells were cultivated in low glucose Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% FBS (PAA) and 1% antibiotic/antimycotic solution (PAA). Culture medium was replaced after every 2-3 days and cells were grown at 37 °C in the presence of 5% CO₂. Firstly, cell culture was continued from day 1 after cell seeding to day 5 before live/dead staining and SEM cell fixation.

Live/dead staining

After 5 days, cell culture of three samples of PCL membranes, which included non-treated, gelatin coating and collagen immobilisation (one for each), were stopped cell culture by removing cell culture medium and washing with PBS for 2-3 times. Live/Dead Cell Double Staining Kit (Sigma, USA) was used to validate cell compatibility of PCL membranes. Solution A (Calcein AM solution) and solution B (propidium iodide solution) were added into each sample with the ration of 2:1 in PBS solution to form assay solution. 100 µl of assay solution was

added into each well and the well was incubated at 37 °C for 15 minutes. Then, cell viability was detected under fluorescence light using a fluorescence microscope with 490 nm excitation for live cells. At 545 nm excitation, only dead cells can be observed.

SEM fixation

After 5 days, three samples of PCL membranes, which included non-treated, gelatin coating and collagen immobilisation (one for each) were taken by removing cell culture medium and washing with PBS for 2-3 times. For primary fixation, 2.5% glutaraldehyde solution (Sigma, USA) was used. All samples were soaked in 2.5% glutaraldehyde solution for 1 hour at 4 °C. After that, samples were washed with distilled water for several times to remove glutaraldehyde. Next, ethanol was used to dehydrate the cells by a series of concentrations: 25%, 50%, 70%, 95%, 100% and 100%. Samples were soaked in each ethanol concentration for 10 minutes. Lastly, samples were washed with distilled water and dried in a desiccator for 1 day before SEM examination.

MTT assay for cell proliferation

Nine modified membranes of 10 mm x 10 mm were sterilized and soaked in cell culture medium before cell culture experiment. L929 mouse fibroblast cells were seeded at the density of 5×10^5 cells/well. Cell culture was continued from day 1 after cell seeding to day 7 for MTT cell proliferation test. Vybrant® MTT Cell Proliferation Assay Kit was purchased from Life technologies (Thermo fisher, USA). For L929 cells, before MTT test the culture medium were replaced to 1000 μ L of fresh clear culture medium. After that 100 μ L of the 12 mM MTT stock solution was added to samples and the samples were incubated at 37°C for 4 hours. Subsequently, 1000 μ L of the SDS-HCl solution were added to each

sample and mixed using the pipette carefully. The samples were incubated at 37 °C in the presence of 5% CO₂ for 18 hours before checking with the microplate reader. Pipette was used to mix the solution in each well before testing. In each well, 210 µL of solution was taken and put into 96-well plate for checking the absorbance by using Tecan Ultra Evolution microplate reader (Switzerland).

6.3.1.8. Statistical Analysis

Sample mean values and standard deviations were determined for MTT assay test. The mean values of the number of cell measurement from the absorbance results were compared with a two-tailed Student's t-test at the significance level of $P < 0.05$.

6.3.2. Results and discussion

6.3.2.1. Plasma study and contact angle analysis

In this study, the 2³ factorial DOE was employed to analyse which plasma treatment conditions would lead to the desired level of hydrophobicity on polymeric membrane, or a contact angle around 40 - 60°. This water contact angle range has been reported as a suitable range for cell attachment [275]. Moreover, plasma modification such as Ar plasma is an important initial step for further modification like collagen immobilisation [206]. As shown in Table 6.4, the Run 2 led to desired contact angle (approximately 40°) in which pure argon gas, a low gas flow rate (100 sccm) and a short treatment time (120 seconds) were used. By using argon plasma, the contact angle of PCL surface can be reduced from 75° to around 40° as shown in Figure 6.10. This might be explained by the different process mechanism of argon and oxygen. While Ar gas directly induced functional group onto surface, O₂ plasma process included etching the surface of

polymer. Etching process may smoothen the surface which will affect contact angle hysteresis [276, 277].

Figure 6.10. Comparison of water contact angle between **a)** non-treated, **b)** Ar plasma treated PCL membrane and **c)** summary of water contact angle between non-treated, O₂ and Ar treated membrane.

Table 6.4. Summary of contact angle results.

Run	A	B	C	Type of gas	Flow rate (sccm)	Argon content (%)	Oxygen content (%)	Treatment time (s)	Contact angle
1	-	-	-	O ₂	100	-	20	120	49.20 ± 4
2	+	-	-	Ar	100	28	-	120	40.56 ± 3
3	-	+	-	O ₂	200	-	40	120	52.19 ± 1
4	+	+	-	Ar	200	56	-	120	41.07 ± 1
5	-	-	+	O ₂	100	-	20	180	53.93 ± 1
6	+	-	+	Ar	100	28	-	180	43.07 ± 3
7	-	+	+	O ₂	200	-	40	180	52.25 ± 3
8	+	+	+	Ar	200	56	-	180	42.21 ± 1

6.3.2.2. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

Figure 6.11 below shows the signal curves of non-treated and surface modified PCL membranes. The ATR-FTIR was used to determine the changes in chemical properties of membrane. The signal follows the same finger print as identified by

Elzein *et al.* (2004) [278]. Thus, it can be confirmed that the solvent-free membrane fabrication did not affect the chemical properties of PCL.

Moreover, as pointed by the arrows, due to the treatment by Ar plasma, peroxides (R-O-OR') and hydroperoxides (R-O-OH) groups were introduced onto the surface, which were in the range of 3200-3600 cm^{-1} . Amine group (1600 cm^{-1} wavenumbers) can be seen after collagen immobilisation, which is similar to Sousa and his colleagues' work (2014) [206].

Figure 6.11. ATR-FTIR curve of the PCL porous membrane in absorbance mode which pink line represented signal from collagen immobilized membrane made, blue line represented signal from gelatin coated membrane, black line represented signal from Ar plasma membrane and green line represented signal from non-treatment PCL membrane made from 500 μm powder.

6.3.2.3. Differential scanning calorimetry (DSC)

In Figure 6.12, the DSC results show that PCL membrane melting temperature (T_m) followed the similar trend as PCL in powder form. However, the Ar treated

membrane required less amount of heat flow and T_m is in the lower range compared to other PCL forms. By coating PCL membrane with gelatin, T_m is shifted to the higher range compared to other forms due to protein functional group on the membrane surface.

Figure 6.12. Heat flow curve from DSC experiment of PCL powder, PCL membrane, PCL membrane with Ar plasma and PCL membrane with gelatin coating.

6.3.2.4. L929 fibroblast cell culture study on surface modified membranes

As shown in Figures 6.13a, 6.13c and 6.13e, for live/dead staining of L929 fibroblast cells at day 5 of cell culture experiment, the results suggest that by integrating natural protein such as gelatin and collagen onto membrane surface, there were more live cells on the coated membrane compared to the non-modified membrane. However, by using live/dead staining, the cell morphology could not be seen clearly. Thus, SEM fixation was used to investigate cell attachment behaviour on non-modified and natural protein surfaced modified membranes.

From SEM image shown in Figure 6.13b, L929 cell morphology was round compared to cell morphology on gelatin coated membrane and collagen immobilized membrane that are shown in Figure 6.13d and 6.13f. Both coated

membranes showed that cells spread widely and there were less dead cells on surfaces especially for the collagen immobilized membrane. This is similar to the work of Cheng *et al.* which also showed the improvement of cell adhesion and cell proliferation for collagen grafted film [204].

Figure 6.13. Live/dead staining of day 5 L929 cells at magnification 4x on **a)** non-modified PCL membrane, **c)** gelatin coated PCL membrane and **e)** collagen immobilized PCL membrane. SEM image of day 5 L929 cells at 1000x **b)** on non-modified PCL membrane **d)** on gelatin coated PCL membrane and **f)** on collagen immobilized PCL membrane.

6.3.2.5. MTT assay results

From MTT results in Figure 6.14, the collagen immobilized PCL membrane has shown better cell proliferation compared to 24-well plate. Even though from day 1 to day 3, number of cells are quite similar between 24-well plate and collagen immobilisation sample, the trend of cell proliferation has significantly changed

in day 7 when the membrane sample has almost double number of cells compared to 24-well plate as Student's t-test indicates that $P < 0.05$. The reason is that the membrane is porous so there is more space for cell to adhere. Secondly, as similar results were reported in the work of Cheng *et al.*, N-group from collagen plays significant role in cell proliferation. Moreover, collagen immobilisation made PCL surface even rougher so the overall area was increased [204]. Therefore, by using collagen type I with optimum plasma condition, surface property of PCL membrane was improved, making it more suitable for cell proliferation and cell adhesion.

Figure 6.14. MTT results of collagen immobilized PCL membrane compared to 24-well plate, *indicates $P < 0.05$.

6.4. Summary

This study has shown that biopolymer like PCL (in membrane form) could be modified to enhance the cell adhesion and proliferation. The membrane was solvent free so it had good cell compatibility and low toxicity. The effect of gas plasma showed that by using Ar plasma at optimum process condition, water

contact angle of PCL membrane could be reduced to 40° and Ar has better surface modification than O₂ plasma. Moreover, with further modification by using the collagen immobilisation techniques, cell proliferation was greatly improved as shown in the MTT test and better than the control group. The objective of this chapter is to investigate and study the modification technique for improving cell adhesion and proliferation on the surface. The results as described above showed that the combination of synthetic polymer with naturally derived polymer provided a good environment and mechanical strength for cell to growth, which could be served as a cell carrier to transfer large amount of cells. In order to improve this work, other parameters such as the blowing process of excess powders, the melting temperature, melting time and the used of porogens should be considered in the future in order to control the porosity of the membrane.

For thick tissue construct, a large amount of cells are needed in order to promote cell differentiation and cell functionality [40, 279]. As described earlier in Chapter 1, the high cell density could be achieved by using cell sheet method, which is a bottom up fabrication approach for tissue engineering. The method in this research could be alternative to cell sheet method for constructing thick tissues. As mentioned earlier, by using collagen immobilized solvent free membrane, the high cell density sheets could be easily cultured and transferred, which is however one major problem in current cell sheet technology. Moreover, the large number of membrane-based cell sheets may be stacked to form an engineered vascularised thick tissue structure by incorporating endothelial cells.

Chapter 7 Conclusions and future Work

7.1. Conclusions

7.1.1. Mathematical modelling of hydrogel printing resolution

A simple analytical mathematical model for estimating hydrogel resolution on extrusion based bioprinter has been established. 24.5 wt% Pluronic F-127 was used as the tested materials and printed via pneumatic extrusion-based bioprinter. The width of the printed hydrogel strand was treated as the hydrogel resolution and was measured against the nozzle size, gauge pressure and stage moving speed. The theoretical data and experimental data aligned with each other in general. However, some experimental errors were still observed due to the capillary effect on the entrance of the nozzle and surface tension of hydrogel. Noticeably, at the high stage moving speed, the errors from capillary effect and surface tension tended to be smaller and seemed negligible. Thus, this analytical model could be used as a resolution prediction tool for the extrusion based bioprinter especially for high viscosity hydrogels like pluronic based hydrogel or other micelles packing based hydrogel.

7.1.2. Hydrogel composite synthesis and *in vitro* evaluation

3D complex hollow structures are required in many biomedical applications from drug testing to vascularization. However, nowadays, there are limited ranges of materials that are suitable for direct 3D complex hollow fabrication onto a solid platform. Thus, a novel material, Plu-GelMA hydrogel composite, has been developed in this work in order to provide both printability and shape integrity. By using the support materials together with the new hydrogel composite, a single step printing of 3D hollow vasculature-like structures has been achieved.

Moreover, from *in vitro* investigation, the Plu-GelMA composite showed a low cytotoxicity since most of the L929 fibroblast cells were alive. The proliferation test shows that the number of cells increased until day 7. Not only supporting cell viability and cell proliferation, Plu-GelMA hydrogel composite could also support cell differentiation and cell attachment as demonstrated in HUVECs experiment are evidenced by two endothelium cell markers, CD31 and VWF, observed on the surface on the hydrogel composite. The presence of the HUVECS markers was a proof for the efficiency of vascularization. This implied that the Plu-GelMA composite had a good potential to be used for vascularization and other TE applications. Lastly, the hybrid structure, which was fabricated from HUVECs cell-laden GelMA hydrogel and the Plu-GelMA, were shown to provide good viability and promote cell alignment. The design intent of the hybrid structure was to mimic the human blood vessel anatomy. Thus, this hybrid structure could be further used and investigated for more vascular and TE applications.

7.1.3. Membrane fabrication and modification for cell sheet transfer

PCL membranes were fabricated from a novel solvent free method, and *in vitro* test showed that they had good cell compatibility but low attachment. Therefore, PCL membranes were further modified in order to improve cell adhesion and cell proliferation. By using argon gas plasma modification at the optimum condition, the water contact angle which represented the hydrophobicity of PCL membrane could be reduced to 40°. Moreover, with a second modification which was collagen immobilisation, the hydrophobicity of PCL membrane could be further reduced. MTT test showed that the cell proliferation has been significantly improved as compared to the nonmodified membranes. These results suggested

that the integration of synthetic biopolymer and naturally derived polymer could provide both mechanical strength and cell-favourable environment. This integrated platform could be used as a cell carrier to transfer tissues or a large number of cells.

As mentioned earlier, in order to fabricate thick tissue construct, a large number of cells are required in order to promote cell proliferation and differentiation or to acquire cell functionality. Cell sheet technology is one solution that is able to provide high cell density. The modified collagen PCL membrane could be another possible solution to be used for cell sheet transfer in order to promote high cell density in the structure of thick tissue constructs. Moreover, PCL membrane had good mechanical properties, which could be convenient to stack and form an engineered vascularised thick structure.

7.2. Future Research Directions

7.2.1. Developing the new hydrogel composite combination

Not only gelatin can be modified into photo-crosslinked hydrogel like GelMA, other naturally derived polymers such as collagen, fibrin and alginate can also be altered and changed into photo-crosslinkable hydrogel. All the mentioned hydrogels have low printability at 37 °C similar to GelMA. Thus, by using pluronic or Plu-MP to integrate with them, it may be possible to create different types of printable hydrogel composites. Moreover, each type of hydrogel is suitable for certain specific applications. For example, collagen is particularly good for skin TE and wound healing compared to other natural polymers. By combining modified pluronic with different natural photo-crosslinkable hydrogels, the range of materials suitable for bioprinting could be greatly increased. Since pluronic could be used to improve the printability of the

hydrogel composite, more complex structures for different TE applications could possibly be fabricated via the extrusion based bioprinting in future.

7.2.2. Co-culture on hybrid structure and perfusion bioreactor

The hybrid structure between cell-laden GelMA hydrogel and Plu-GelMA hydrogel composite has shown good potential for achieving cell viability and cell alignment of vascular cells. To further investigate vascularization efficiency, the hybrid structure should contain more than one type of cell. Human fibroblast and human endothelial cells could be used for co-culture since both of them are the cells forming blood vessel [280]. The strategy can be implemented in two different ways. Firstly, fibroblasts and endothelial cells can be mixed and put inside GelMA for hybrid structure fabrication. In the second method, endothelial cells are put inside GelMA and get printed first and then the fibroblast cells are seeded onto the printed structure. The combination of both techniques can also be implemented, for example, there is a research work that combined both top down and bottom up approaches for cell culture of bioprinted structure [77]. In order to fully investigate the development of vascularization, it is necessary to put the printed structure in a perfusion bioreactor for long-term culture and long-term study of the functionality of an engineered 3D thick vascularized tissue construct.

7.2.3. Combination of thermo-responsive hydrogel on membrane for cell sheet transfer system

In order to further advance cell sheet transfer system, the thermo-responsive hydrogel should be used as the first coated layer before collagen immobilisation. In this case, the collagen and cell sheet can be transferred by using the membrane and can be easily separated when the temperature is increased to 37 °C. Thus, the

first layer should be agarose or gelatin that can be easily melted away at a higher temperature range ($>35^{\circ}\text{C}$). Recently, a new technique that turns cell sheets into a new bioink has been reported [281]. By using a collagen immobilized membrane, a large amount of cell sheets could possibly be generated at the same time. Thus, PCL membranes can be used as a platform for generating cell sheets to develop new types of bioink.

7.3. List of publications

7.3.1. Patents

R. Suntornnond, J. An, C.K. Chua., Y.S. E. Tan, W.Y. Yeong and J.K. Er (2017)
“Free Standing Hollow 3D Bioprinted Structure From Highly Printable Bio-Ink”,
Singapore Patent, Provisional Application No. 10201701620X.

7.3.2. Journal Papers

1. R. Suntornnond, Y.S. E. Tan, J. An, and C.K. Chua. “A highly printable and biocompatible hydrogel composite for direct printing of soft and perfusable vasculature-like structure”, *Scientific Report*, 7 (1), 16902 (2017).
2. R. Suntornnond, J. An, and C.K. Chua. “Roles of support materials in 3D bioprinting – Present and Future”, *International Journal of Bioprinting*, 3(1), 1-4 (2017).
3. R. Suntornnond, J. An, and C.K. Chua. “Bioprinting of thermo-responsive hydrogels for next generation tissue engineering: A review”, *Macromolecular Materials and Engineering*, 302, 1600266 (2017).
4. R. Suntornnond, Y.S. E. Tan, J. An, and C.K. Chua. “A mathematical model on the resolution of extrusion bioprinting for development of new bioinks”, *Materials*, 9(9), 756 (2016).
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6. R. Suntornnond, J. An, W.Y. Yeong and C.K. Chua. “Effect of Gas Plasma on Polycaprolactone (PCL) Membrane Wettability and Collagen Type I

Immobilized for Enhancing Cell Proliferation”, *Materials Letters*, 171, 293-296 (2016).

7. J. An, J.E.M. Teoh, R. Suntornnond and C.K. Chua. “Design and 3D Printing of Scaffolds and Tissues”, *Engineering*, 1(2), 261-268 (2015).
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7.3.3. International conference papers

1. R. Suntornnond, J. Y. Lee, J. An and C. K. Chua. “Bioprinting of thermo-responsive and photo-curable hydrogels for multilayer vessel-like structures”, International Conference on Biofabrication 2017, 15 – 18 October 2017, Beijing, China.
2. R. Suntornnond, J. An, and C.K. Chua. “A Preliminary Study on the Extrusion Resolution of Pluronic F127 for Bioprinting Thermo-responsive Hydrogel Constructs”, 2nd International Conference on Progress in Additive Manufacturing, Pro-AM 2016, 16 – 19 May 2016, Singapore.
3. R. Suntornnond, J. An, W.Y. Yeong and C.K. Chua. “3D Hybrid Membrane Based Structure: A Novel Approach for Tissue Engineering Scaffold”, the 4th International Conference on Additive Manufacturing and Bio-Manufacturing, ICAM-BM, 12-14 November 2014, Beijing, China.
4. Y.S. E. Tan, R. Suntornnond, J. An, W.Y. Yeong and C.K. Chua. “Selective Grafting of Gelatin on Polycaprolactone Membrane for Retinal Tissue Engineering”, the 4th International Conference on Additive Manufacturing and Bio-Manufacturing, ICAM-BM, 12-14 November 2014, Beijing, China.
5. J. An, R. Suntornnond and C.K. Chua. “Bioprinting of Hydrogel on Basement Membrane-Like Polymer Substrate for Engineering 3D Organized Tissue

- Layers”, the 4th International Conference on Additive Manufacturing and Bio-Manufacturing, ICAM-BM, 12-14 November 2014, Beijing, China.
6. R. Suntornnond, J. An, W.Y. Yeong and C.K. Chua. “Organic Solvent-Free Biodegradable Polymeric Membrane Fabrication: A Potential for Antibacterial and Anti-Adhesion Applications”, International Conference on challenges in IT, Engineering and Technology, ICCIET 2014, 17-18 July 2014, Phuket, Thailand.
 7. R. Suntornnond, J. An, W.Y. Yeong and C.K. Chua. “Effect of powder particle size on solvent free membrane for 3D hybrid scaffold structure”, 1st International Conference on Progress in Additive Manufacturing, Pro-AM 2014, 26 – 28 May 2014, Singapore.

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Appendix A: FITC-dextran concentration standard curve **Figure A.1.** Standard curve for FITC-dextran concentration and curve fitting

Appendix B: PrestoBlue® L929 cells standard curve **Figure B.1.** Standard curve for L929 fibroblast cells with curve fittings

Appendix C: Gas content percentage calculation for plasma treatment

Percentage of gas desired can be calculated using the following governing formula for flow rate:

$$\text{Ar: } \frac{x}{100\%} \times (1.44) \times 250 = 50$$

(Where x is the percentage desired)

$$x = 14\%$$

$$\text{O}_2: \frac{x}{100\%} \times (0.994) \times 500 = 50$$

$$x = 10\%$$