Study of hydrogels for 3D printing of constructs with strong interfacial bonding

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STUDY OF HYDROGELS FOR 3D PRINTING OF CONSTRUCTS WITH STRONG INTERFACIAL BONDING

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Abstract

Hydrogels are the most appealing candidates of biomaterials for bioprinting. In this field of bioprinting, the lack of suitable hydrogels remains a major challenge. Thus, choosing appropriate hydrogels is the key to successfully print self-supporting 3D constructs. Most importantly, the design criteria regarding the bioinks and the obtained constructs should be made clear in advance. Therefore, the first task of this study is to clarify the design criteria regarding the important properties of a potential bioink and the generated 3D construct, including rheological, interfacial, structural, biological, and degradation properties, which are crucial for printing of complex and functional 3D structures. A method is developed for evaluating the printability of a candidate hydrogel through simulating its rheological behaviors before, during, and after printing. After that, two novel strategies are proposed in order to obtain multilayered hydrogel constructs with strong interface bonding. In the first strategy, trisodium citrate (TSC) acts as a chelating agent to remove the superficial Ca^{2+} at each layer. The subsequent post-crosslinking of constructs in a calcium chloride bath will further create the crosslinks and enhance the adhesion between adjacent layers. The second strategy for improving the adhesion between printed layers of a construct is to exploit the interaction between two oppositely charged hydrogels. On the basis of these two strategies, the exciting results have been obtained, which include strong interfacial bonding between two layers of the printed structures, good shape fidelity

of the printed constructs, suitable structural integrity of the constructs, and excellent biocompatibility for the bioprinted constructs. It is hoped that the above-mentioned specific considerations for 3D printable hydrogels and their 3D printed constructs could help the researchers in selecting or developing a suitable hydrogel for bioprinting.

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Publications

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List of Abbreviation and Symbols

Abbreviation and symbols	Description	Unit
Alg	Alginate	—
RGD	Arginine-glycine-aspartic acid	—
A	Area of a grid	mm ²
Chi	Chitosan	—
C	Circularity of an enclosed area	—
Cg	Critical gel concentration	wt %
DI water	Deionized water	—
DM	Degree of methacrylation	—
DMEM	Dulbecco's modified Eagle's medium	—
DPBS	Dulbecco's phosphate-buffered saline without calcium and magnesium	—
EDTA	Ethylenediaminetetraacetic acid	—
ECM	Extracellular matrix	—
FBS	Fetal bovine serum	—
<i>u</i>	Flow rate	mm/s
Gel	Gelatin	—
GelMA	Gelatin methacrylate	—
GO	Graphene oxide	—
HBSS	Hanks' balanced salt solution without calcium and magnesium	—
I.D.	Inner diameter	mm

D_1	Inner diameter of the syringe	mm
D_2	Inner diameter of the nozzle	mm
Kca	κ -Carrageenan	—
L	Length of an element	—
<i>LIFT</i>	Laser induced forward transfer	
D_L	Line distance	mm
MC	Methylcellulose	—
M_w	Molecular weight	—
NMR	Nuclear magnetic resonance	—
OM	Optimal microscope	—
m	Power-law consistency coefficient	—
n	Power-law index	—
P	Pressure	Pa
P_L	Perimeter of a grid	mm
ΔP	Pressure drop	—
W_r	Percentage of degradation	—
P_r	Printability	—
PI	Photo initiator	—
R	Radius	mm
R_1	Radius of the syringe	mm
R_2	Radius of the nozzle	mm

SEM	Scanning electron microscope	—
$\dot{\gamma}$	Shear rate	s^{-1}
$\dot{\gamma}_1$	Shear rate in the syringe	s^{-1}
$\dot{\gamma}_2$	Shear rate in the nozzle	s^{-1}
τ	Shear stress	Pa
V_1	Speed of the piston	mm/s
V_2	Speed of the extruded hydrogel in the nozzle	mm/s
S.D.	Standard deviation	—
3D	Three-dimensional	—
TCPS	Tissue culture polystyrene	—
TE	Tissue engineering	—
TSC	Trisodium citrate	—
USS	Ultimate shear stress	Pa
V	Uniform flow rate	mm/s
η	Viscosity	Pa·s
Q	Volumetric flow rate	mm^3/s
W_0	Weight of a hydrogel before soaking	g
W_1	Weight of a hydrogel after soaking	g
Xan	Xanthan	—

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Chapter 1 Introduction

1.1 Background

The recent statistics from U.S. Department of Health & Human services reported that more than 116, 000 patients were on the national transplant waiting list as of August 2017 [1]. Although organ transplantation has showed remarkable achievements in saving lives, an average of 20 people died each day in the U.S. alone in 2017 while waiting for a transplant [1]. In the early 1970s, tissue engineering (TE) was introduced based on the demands for organ transplantation and shortage of available donors [2].

One disadvantage of traditional approaches for fabricating artificial scaffolds is the lack of the complexity of native tissue or organs [3]. Meanwhile, the artificial tissues or organs could not transport nutrients and exchange oxygen without an appropriate porous structure and an interconnective complex geometry. Moreover, cells are generally randomly deposited when utilizing the traditional TE fabrication approaches [4]. To overcome the above obstacles, three-dimensional (3D) printing technologies have been developed which are primarily technologies to fabricate 3D cell-laden constructs. Specially, in the area of TE, 3D bioprinting technology enables us to fabricate tailor-made tissues with patient-specific geometry through precisely controlled deposition of a bioink, which is a mixture of a biomaterial and living cells [5]. Due to the tremendous potential of 3D printing technologies [6-8], the progress

in this field is very rapid over the last decade.

1.2 Problem statement

Most of hydrogels are appealing candidates for 3D bioprinting because they are biocompatible and could provide 3D environment with a highly water content. Historically, natural hydrogels are commonly used for TE [9-11], including alginate [12-14], collagen [15], gelatin [13, 16], and chitosan [17, 18]. However, it is very challenging to stack a natural hydrogel into a 3D construct because natural hydrogels are weak by nature and cannot support the 3D structure without collapsing. In contrast, synthetic hydrogels can be tailored with robust mechanical properties [9, 19]. However, synthetic hydrogels are often of a poor biocompatibility and produce non-natural degradation products [9, 19]. The development of a robust hydrogel for bioprinting, which is suitable for both bioprinting and cell culturing, is still a challenge [4].

Thus, the key task for printing a 3D construct is to choose an appropriate hydrogel for making a bioink. Before that, the specifications or criteria for a candidate hydrogel for 3D printing should be made clear. The specifications should include not only for the bioinks themselves for printing, but also for the obtained bio-printed constructs for a desired TE application. This knowledge is useful for the researchers in selecting or preparing a suitable hydrogel for bioprinting.

To successfully obtain a 3D construct for bio-application, the specific

considerations regarding the important properties of a candidate hydrogel and the generated 3D structure, can be classified into two groups: during printing, and after printing. During a printing process, the candidate hydrogel should exhibit a shear-thinning behavior, implying that a viscoelastic hydrogel could be easily extruded out through a fine nozzle. After printing, i) the hydrogel should be highly thixotropic, meaning that the shear-reduced viscosity could quickly recover, and then the extruded filament could have sufficient mechanical strength to maintain its shape and then support the subsequently printed layers. ii) There are layer defects in the 3D printed constructs due to the layer-by-layer printing process. Therefore, the interfacial properties between the printed layers should be examined. iii) The hydrogel for printing should be able to generate a 3D complex construct with a high stackability and a high shape fidelity. iv) The printed structure should be biocompatible. v) The bioprinted construct is degradable, but the structure must be stable in vitro culture or in vivo environments over a desired time according to its applications.

Among all the considerations, interfacial bonding is one of the important considerations for successfully obtaining a 3D structure. However, it is rarely mentioned in the previous literature [20, 21]. Rheology is the study of the flow behavior of materials under application of an external force or deformation, which is highly relevant to a bioprinting process. All the previous works[12, 22, 23] indicate that rheological properties of a candidate hydrogel are important in controlling the 3D printability of the hydrogel. However, the relation of a 3D printing process with the

rheological behavior of a candidate hydrogel and the resultant quality of a printed 3D structure are still not clear. The importance of rheological considerations for 3D printing is often underestimated [4]. It is needed to clearly demonstrate the relation between 3D printing process (before, during, and after printing) and the rheological behavior of a candidate hydrogel.

1.3 Objectives and scope

This study aims to address the challenges in selecting hydrogels for 3D printing constructs for biomedical applications as discussed in Section 1.2. Thus, the goal of this research is to select a suitable hydrogel and then successfully print 3D constructs for biomedical application with the necessary considerations, focusing on the two main aspects: i) finding out a suitable hydrogel for 3D printing on the basis of developing a rheological approach to evaluate the printability of a candidate hydrogel; and ii) developing strategies for printing 3D structures with strong interfacial bonding.

The main objectives are listed as follows:

- i) To present a novel rheological approach to simulate the rheological behaviors of a candidate hydrogel before, during, and after the 3D printing process; and estimate the printability of this hydrogel through rheological measurement.
- ii) To develop the strategies for printing a 3D hydrogel constructs with strong interfacial bonding between the printed layers.

1.4 Thesis outline

The report consists of six chapters as outlined as follows:

Chapter 1 gives a brief introduction to the background and the motivation of this study. The objectives and scope of this dissertation are stated.

Chapter 2 reviews the current technologies for 3D bioprinting, as well as the current hydrogels used for bioprinting and their limitations. Most importantly, the considerations regarding the suitable bioinks and the printed 3D constructs are discussed and highlighted.

Chapter 3 presents an equation for estimating the value of shear rate exerted on a hydrogel during the extrusion process. A novel approach is developed for simulating the rheological behaviors of a candidate hydrogel before, during, and after the printing process. Alginate is selected as a model material to demonstrate this approach and its printability is evaluated. Finally, the effect of graphene oxide on the printability of the alginate-based hydrogels is discussed.

In Chapter 4, a novel and robust alginate / methylcellulose blend hydrogel with a smart strategy to improve interfacial adhesion between printed layers, is developed for 3D bioprinting. In this work, trisodium citrate (TSC) possess two functions: an interfacial bonding improving agent, and a bioink medium for loading cells for 3D bioprinting. A parametric study is carried out to determine the key factors that affect the adhesion at the interface of the layered hydrogels structure. Rheological properties

of the blend with different formula are investigated, simulating their rheological behaviors before, during, and after printing. Lap-shear test is conducted to evaluate the interfacial bonding strength between the printed layers. Meanwhile, the effectiveness of this pair of bioink (the blend hydrogel and the TSC solution) is investigated, including 3D printability, mechanical properties, degradation behavior, and in vitro biocompatibility.

In Chapter 5, a novel strategy for improving the adhesion between printed layers of a 3D printed hydrogel construct is developed by smartly exploiting the interaction between two oppositely charged hydrogels. Three anionic hydrogels (alginate, xanthan, and k-carrageenan) and three cationic hydrogels (chitosan, gelatin, and gelatin methacrylate) are chosen to find the best combination of two oppositely charged hydrogels for the best printability with strong interfacial bonding. Rheological properties and printability of the hydrogels, as well as structural integrity of the printed structure in the cell culture medium, are studied as functions of the polymer concentration and the combination of hydrogels.

Chapter 6 summarizes the present research work and a brief suggestion for the future work is also presented in this chapter.

Chapter 2 Literature Review

This chapter provides a literature review on the technologies for bioprinting, and the current hydrogels used for bioprinting and their limitations. Most importantly, the considerations regarding the suitable bioinks for 3D printing and the generated constructs for biomedical applications are discussed.

2.1 Bioprinting technologies

3D printing technologies are developed to print 3D structures through layer-by-layer stacking of materials in a pre-designed pattern. Stereolithography is the first 3D printing technology, which was introduced in 1986 and commercialized later [24, 25]. After that, the concept of fused deposition modeling was patented in 1992. But, the high cost and complexity of these early technologies limited their application. In the past decades, the commercialization of the cost-effective 3D printers has broadened the application of these technologies, including tissue engineering, architectures, toy industries etc. For bioprinting of a 3D construct, a 3D printing technology and a bioink are the key elements [26].

An overview of the commonly used 3D printing technologies for bio-fabrication is presented below. On the basis of the printing mechanisms, bioprinting technologies could be classified into three groups: extrusion printing, inkjet bioprinting, and laser induced forward transfer (LIFT) [4, 26]. As shown in Table 2.1, these three printing methods are compared from different aspects, such as materials, structure, processing,

and cost. In the following section, the more detailed information is given for each fabrication technology.

Table 2.1 Comparison of 3D bioprinting technologies

Method	Extrusion printing	Inkjet	LIFT	References
Material viscosity	High (30-6×10 ⁷ mPa·s)	Very low (3.5-12 mPa·s)	Low (1-300 mPa·s)	[7, 27, 28]
Cell viability	40-95%	>85%	>95 %	[4, 7, 29, 30]
Cell densities	High cell spheroids	Low <10 ⁶ cell/mL	Medium ~10 ⁸ cells/mL	[29, 31-33]
Working principle	Contact	Noncontact	Noncontact	[34]
Size of nozzle	20 µm -1 mm	20-150 µm	Nozzle free	[35, 36]
Resolution	20 µm	50-300 µm	20-80 µm	[7, 9, 34, 37-39]
Structure thickness	Vertical thick	Very thin	Thin	[40]
Printer cost	Medium	Low	High	[27]

2.1.1 Extrusion printing

In an extrusion printer, bioink is generally loaded into a disposable plastic syringe, and then extruded out through a nozzle. Pneumatic- or mechanical-driven dispensing is employed for an extrusion printer. Mechanically driven dispensing includes piston-driven or screw-driven (Figure 2.1). In a pneumatic-driven system, the valve triggering bioink ejection sits between the bioink and the inlet of the pressurized air

[34]. In a piston-driven system, a bioink is extruded by pushing a piston. The piston-driven printing approach generally gives more directly control over the bioinks, comparing to a pneumatic-driven printer for which there is a delay of the compression gas volume. In a screw-driven system, rotation of the screw extrudes the bioink from the syringe to the nozzle. The feeding of bioink is controlled by the rotation speed of the screw and the design of the screw [4]. Screw-driven dispensing is beneficial for printing of bioinks with high viscosity. But the larger pressure drops at the nozzle which generates from the screw-driven dispensing, has potential harm on cells. In piston-based or pneumatic dispensing systems, cells are printed with high cell viability [26, 34]. All extrusion printing systems can produce continuous filaments through a fine nozzle, rather than single drops.

Figure 2.1 Schematic illustration of three extrusion printing systems [4].

To obtain a 3D construct with a good shape fidelity, highly viscous bioinks are commonly used. The viscosities of bioinks for extrusion printing have a wide range

($30\text{-}6\times 10^7$ mPa·s) [26, 34]. The resolution of extrusion printing can be achieved to about 20 μm [39, 41]. The printing speed of extrusion printing is significantly higher than that of the inkjet printing. Bioinks used for extrusion printing are primarily hydrogels, including alginate [12-14, 42], fibrin [43], collagen [44], gelatin methacrylate (GelMA) [45, 46], and hydrogel blends etc [47, 48].

The extrusion printing is already regarded as the most promising technology due to the fast printing speed and capability of fabrication of clinically-relevant sizes of 3D constructs [34]. To date, the fabrication of thick 3D constructs (see Figure 2.2 (a) and (b)) [47, 49], and vascularized tissues [50-52] (see Figure 2.2 (c)) has been achieved using extrusion printing. Gao et al., [50] reported a strategy to fabricate large-scale organs with built-in microchannels, as shown in Figure 2.2 (c). The image on the left side of Figure 2.2 (c1) illustrated the designed coaxial nozzle-assisted bioprinting system. In this system, a 2% alginate solution and a 4% CaCl_2 solution were co-extruded with different flow rates to print the hollow alginate filament, as shown on the right side of Figure 2.2 (c2). The printed filament demonstrated an average inner diameter of 892 μm . Furthermore, a perfusion test was conducted by pumping the cell culture media into the printed hollow filament, which proved the printed microchannel without any occlusion issue (Figure 2.2 (c3)).

Figure 2.2 Demonstration of the printed structures. (a) 20 layers scaffold using the mixture of gelatin, alginate and collagen [47]. (b) 3D printing of tall structures using the mixture of alginate and gelatin with a proper rate [49]. (c) Schematic illustration of the coaxial extrusion process [52]. Images on the right side show that the printed hollow filament is under a perfusion test [50].

2.1.1.1 Advantages

In terms of some of the favorable capabilities such as the ability of printing highly viscous bioinks (6×10^7 mPa·s) [27] with a good cell viability [4], and the ability of fabricating thick 3D construct [4, 53], extrusion printing exceeds inkjet bioprinting.

2.1.1.2 Challenges

A major challenge for extrusion printing is the obligation of using a narrow nozzle, which requires a high driving pressure or large extruding force applied on a hydrogel-based bioink. The high printing pressure or large extruding force exerted on the cell-

laden hydrogel has a negative effect on cell viability due to the high nozzle shear forces [23], especially when the highly viscous bioinks are used. The aim of obtaining a construct with a high cell viability could be achieved by decreasing the printing pressure, extruding force, or increasing the nozzle size. However, a corresponding lower resolution is obtained by utilizing a nozzle with a bigger diameter. Additionally, the tendency of nozzle clogging is an intrinsic problem when using a highly viscous bioink. Ghorbanian et al. introduced a microfluidic direct writer, which included a de-clogging mechanism of using a solvent to dissolve the clogging materials [54].

2.1.2 Inkjet bioprinting

Inkjet bioprinting is generally defined as dispensing of very small volumes (1-100 picolitres) of a low viscosity bioink on to a substrate [55]. The inkjet printer utilizes a thermal [29] or piezoelectric [56, 57] actuator as a driving force to deposit droplets in a designed pattern. The printer can be generally operated in two modes: drop-on-demand inkjet bioprinting and continuous inkjet bioprinting [34], as shown in Figure 2.3 (a). For a thermally-induced inkjet printer, the droplets are generated through heating. A heater is utilized to heat and evaporate the surrounding bioink, and the generated vapor bubble will expand rapidly to expel bioink droplets out from the printing head [58]. For a piezoelectrically-induced inkjet printer, bioink is extruded from the chamber using piezoelectric actuators after a pulse is applied. The applied voltage will cause the generation of a pressure wave, which leads to the ejection of

droplets [59]. Inkjet printing has been employed for fabrication of multilayered droplets (Figure 2.3 (b)) [60], and even a small 3D structure (Figure 2.3 (c)) [60].

Figure 2.3 Inkjet printing. (a) Schematic drawing of inkjet printing [4].(b) Hydrogel droplets with different layers (up to 5 layers) [60]. (c) A printed triangle hydrogel structure with 10 layers [60].

2.1.2.1 Advantages

The advantages of inkjet printing include the high spatial resolution (50-300 μm) [7, 37], high printing speeds (up to 10000 drops /s) [4, 7], and low cost [7, 61]. In addition, the modification of commercially available inkjet printers into 3D bioprinters is a cost-effective way utilized in many labs to fabricate tissue constructs.

2.1.2.2 Challenges

For inkjet bioprinting, there are many challenges, including the ejection of liquid phase bioinks, only a limited range of viscosity (3.5-12 mPa·s) for printable bioinks [7, 26], and a restriction to thin constructs due to a discretized flow [40]. In a thermal inkjet bioprinter, the applied heat causes evaporation leading to transient pores in the cell membrane, as the temperature in the nozzle could reach 300 °C or even higher [29, 62]. There is a risk of cells' lysis in the high frequency range of piezoelectric pulses and also the excessive thermal stress might impact the functionality of cells and cell viability [26]. Lastly, when depositing a line or a 3D structure, the interaction and gap between two adjacent droplets should be considered. The surface energy is also crucial for the interactions between two droplets, and the droplets should be stable enough to maintain their shapes before solidification, and then form a uniform line or a structure [63].

In summary, the inkjet bioprinting technology faces a challenge to print larger, more complex and clinically relevant sizes of constructs for bio-fabrication, because this technology only generates continuous small droplets [4, 63].

2.1.3 Laser induced forward transfer

Laser induced forward transfer technology utilizes the laser energy for printing of bio constructs [64]. In 1999, Odde et al., firstly demonstrated to accurately deposit the biological materials (i.e., fibronectin) and individual cells into clusters to fabricate a

3D construct [65]. After that, this technology was extensively utilized to deposit biological materials, including DNA [66], peptides [67], and living cells [68]. The printer system contains three components: a donor slide, a laser source that produces a pulsed laser beam, and a laser absorbing layer [68], as illustrated in Figure 2.4. One layer of the bioink is placed on the bottom of the donor slide. During the bioprinting process, a laser pulse is applied on the donor layer, which leads to generation of a high-pressure and a microscale bubble. The bubble expands to expel the bioink droplets onto the substrate [26].

Figure 2.4 Schematic illustration of the mechanism of the laser induced forward transfer [4].

2.1.3.1 Advantages

This non-contact bioprinting approach has several advantages. Firstly, there is no contact between the bioinks and the dispenser, which leads to a decreasing chance of contamination. Moreover, a highly viscous bioink can be used and the viability of

cells is high (>95%) because no mechanical forces are directly applied on the cells [69]. Additionally, this technology can generate high resolution droplets (20-80 μm) [7, 26, 38], and bioinks with a high cell density (10^8 cells/ mL) [7] can be utilized.

2.1.3.2 Challenges

A relatively narrow range of printable viscosities (1-300 mPa·s) [4, 26, 34] are utilized for laser induced forward transfer. Furthermore, the method is not commonly employed in many labs due to the complexity of laser pulse control and the high cost of laser sources [26].

2.2 Current hydrogels for biofabrication and their limitations

Hydrogels for biofabrication can be natural or synthetic. Naturally derived polymers for hydrogels, including alginate [12-14], collagen [15], gelatin [13, 16], and chitosan [17, 18], which show the good biocompatibility, have received great attention. However, these hydrogels have limitations for 3D printing because they are weak by nature [9]. Synthetic derived hydrogels (e.g. polyacrylamide, poly (vin yl alcohol), and poly (2-hydroxyethyl methacrylate), can also have good or acceptable biocompatibility, but their degradation products may be a concern [9, 19]. Furthermore, during a 3D printing process, UV exposure for chemical cross-linking of a UV-curable polymer may harm cells. Developing a robust hydrogel that is suitable for both bioprinting and cell culturing, is urgently needed [4]. In this section, several popularly used hydrogels for 3D printing will be compared. Their limitations for 3D

bio fabrication are also discussed.

2.2.1 Alginate

Alginate is an anionic polysaccharide typically derived from various species of brown seaweed [70]. Alginate consists of blocks of (1→4)-linked β -D-mannuronate (M) and α -L-guluronate (G) residues. Within an alginate chain, the blocks are composed of repeating M residues, consecutive G residues, and alternating G and M residues [70, 71]. The chemical structure of alginate is shown in Figure 2.5. According to Gacesa et al.,[72] the G-blocks are stiffer and more extended in chain configuration than M-blocks. It is because there is a higher degree of hindered rotation around the glycosidic linkages. Meanwhile, alginates rich in M-blocks form soft and elastic hydrogels, whereas those rich in G-blocks form hard and brittle hydrogels. Moreover, alginate can form hydrogels with the divalent cations such as Ca^{2+} , Ba^{2+} and Mg^{2+} [73, 74]. It is believed that G-blocks makes the major contribution to form hydrogels through intermolecular ionic cross-linking with the divalent cations, because the structure of G-block allows a high degree of coordination of divalent cations [71, 75]. Additionally, the crosslinking density and then the mechanical properties of the ionically crosslinked alginate hydrogels could be controlled by varying the ratio of G to M in the alginate polymer [76]. Thus, the viscosity of alginate hydrogels depends on the average molecular weight (M_w) of alginate, the concentration of alginate, and the ratio of G to M in the alginate polymer [77].

Figure 2.5 Chemical structure of G-block, M-block, and GM block in alginate[75].

Alginate hydrogels can be formed through different mechanisms. Alginate's chains contain negatively charged carboxylate groups, which can form ionic crosslinks with the positively charged cations (i.e., Ca^{2+} , Ba^{2+} , Mg^{2+}) [59]. In addition, alginate hydrogels can also be prepared by covalent crosslinking with poly (ethylene glycol)-diamines [9]. However, the unreacted chemicals need to be completely removed from the hydrogels because the covalent crosslinking reagents may be toxic [75].

Alginate is one of the most frequently used hydrogels in a variety of bio-applications, because of its favorable biocompatibility, relatively low cost, and ease to be used for printing [75]. But there are limitations of alginate hydrogels for printing. Firstly, alginate is mechanically weak so that the printed structures with pure alginate

hydrogels cannot maintain the pre-designed pattern [78]. A common approach to improve mechanical strength of alginate hydrogels is by increasing the concentration of alginate, which results in a better quality of printing. The physical cross-linking agent such as CaCl_2 is usually used for improving the shape fidelity of a printed structure through forming ionic cross-linking with alginate [42, 79]. An example for preparation of alginate hydrogels is given by Tabriz et. al. [42]. They prepared alginate aqueous solutions with various concentrations. The alginate solutions were then mixed with CaCl_2 solutions of various concentrations (i.e. 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 mM, respectively) at a volume ratio of 1:1. The optimal composition for each hydrogel is determined by a self-supporting ability measurement. They found that the minimum alginate concentration which required for forming a self-supporting structure was 4% (w/v) alginate with 40 mM CaCl_2 , as shown in Figure 2.6. Figure 2.6 (a) demonstrated the hydrogel samples with various alginate concentrations. The images show the flow behavior of each hydrogel upon post-transposing the hydrogel-containing tubes. The hydrogels with lower alginate concentrations exhibited an induced flow by gravity, which indicated the low viscosity of alginate hydrogels. The structures printed with various alginate concentrations were shown in Figure 2.6 (b). It was found that the shape fidelity of the 3D structure was improved with a higher alginate concentration. However, the printed 3D structure only had a limited height (0.8 mm, 10 layers, the extrusion nozzle with a diameter $\sim 210\ \mu\text{m}$) [80], which was fabricated with alginate hydrogel of 10%

concentration. This was due to the poor stackability of alginate hydrogels. Furthermore, a critical disadvantage of the ionically crosslinked alginate hydrogel is the short time stability of the printed scaffolds. The short time stability is because the ionically crosslinked alginate hydrogels can be gradually dissolved by releasing of the divalent ions into the surrounding cell culture media through the exchange reactions with monovalent cations (e.g. Na^+). Thus, the mechanical stiffness of alginate hydrogels will be gradually lost over time [75, 81]. Jia et al., [12] found that the alginate hydrogel's mechanical properties were quickly lost (40% within 9 days) during in vitro culture at 37 °C.

Additionally, although alginate is a favorable hydrogel for bio-fabrication, further modifications are often needed to achieve the desirable cellular functions since alginate inherently lacks mammalian cell-adhesivity [75]. It was reported that proteins were minimally adsorbed because of the highly hydrophilic nature of alginate hydrogel [82], which resulted in the poor cellular adhesion and the limited capacity for cells proliferation [47]. This drawback could be overcome by modifying the alginate surface with peptides (e.g. arginine-glycine-aspartic acid (RGD)), which can provide the molecule binding site for cell adhesion [75, 82].

Figure 2.6 (a) Images of alginate hydrogels with different concentrations of alginate in the tubes. (b) 3D printed hydrogel constructs with different concentrations of alginate, showing the self-supporting ability.[42]

2.2.2 Gelatin

Gelatin is a water-soluble protein, which is derived from collagen [83]. Gelatin promotes cell adhesion and proliferation as it retains the RGD sequence from collagen [84]. At the physiological temperature (37 °C), gelatin dissolves as a colloidal sol. But it can form a gel when the temperature decreases (<29 °C) [77]. This is because a conformational change from a random coil structure to a helix structure, which results in chains' association, and eventually the formation of a 3D network [85-87]. Thus, gelatin is rarely considered as a candidate for 3D bioprinting without any prior treatment (e.g. chemical and physical cross-linking) [88]. Much attentions have been paid to overcome this drawback. For example, Chung and his group prepared gelatin-alginate blends, and compared the compression modulus of pure alginate hydrogels (Figure 2.7 (a)) and gelatin-alginate blend hydrogels (Figure 2.7 (b)) over 14 days [77]. In their study, the degradation behavior of all samples in a cell culture medium over the time was observed through the changes in compression modulus (see Figure 2.7). Although the gelatin-alginate blend samples were already post cross-linked in a 2% CaCl₂ solution, this treatment still cannot stop the dissociation of gelatin network at 37 °C. Additionally, the degradation speed of gelatin-alginate blend hydrogels was faster than that of the pure alginate hydrogels. To avoid the liquification of gelatin hydrogels at 37 °C, GelMA is commonly used to obtain a stable 3D structure by exposing the hydrogel to UV light to form the covalent crosslinks of GelMA chains [46, 89, 90].

Figure 2.7 Compression modulus of (a) pure alginate hydrogels with various concentrations, and (b) gelatin- alginate blend hydrogels with different concentrations in cell culture medium up to 14 days at 37 °C. Three concentrations for the pure alginate solutions (1, 2 and 4% w/v), and concentration for the pure gelatin solution was fixed at 10% w/v. All the blends were prepared in the ratio of 4 parts alginate solution: 1 part gelatin solution [77].

2.2.3 Chitosan

Chitosan is a linear polysaccharide, which can be easily derived from the partial deacetylation of chitin [91]. It is poorly soluble in aqueous solutions when $\text{pH} > 7$, but it becomes soluble in the dilute acids ($\text{pH} < 6$) [92]. Chitosan is widely used in tissue engineering, including wound dressing, cartilage regeneration, and fabrication of

sponge scaffolds [93]. Chitosan is the only positively charged natural polysaccharide [94], which is the main mechanisms for chitosan to exhibit haemostatic and antibacterial activities. Chitosan exhibits a haemostatic activity through the membranes of the red blood cells which possesses the negative charge and can interact with the positively charged chitosan [95, 96]. Chitosan can also interact with negatively charged groups at the surface of cells, which might prevent other materials to enter the cells [93]. Finally, chitosan is a biodegradable polymer because it contains breakable glycosidic bonds [93]. Chitosan is able to form a physically associated hydrogel via hydrogen bonds or hydrophobic interactions, all by itself [97]. It can also form hydrogels with negatively charged polysaccharides (e.g. alginate, xanthan) or proteins (e.g. gelatin). However, the weak mechanical strength of chitosan scaffolds limits their application [18]. The previous studies focused on improving mechanical properties of chitosan-based hydrogels through formation of chemical crosslinks, blending with synthetic polymers [98] or making composites with reinforcement agents [99]. The obtained hydrogels could be much stable than the original chitosan hydrogels. However, if any chemical method is used for modification of the primary structure of chitosan, it may potentially change the inherent properties of chitosan. Moreover, a chemical reaction involved might be a source of contamination because the residual reactants may be toxic [93, 100]. In conclusion, chitosan is not suitable for fabrication of large scale scaffolds [17].

2.2.4 Collagen

Collagen is a biocompatible protein, which is one of the principle components of connective tissues in mammals [101]. In the past decades, it has been widely used in biomedical applications, including tissue replacement and regeneration, contact lens, drug delivery, etc. due to its favorable biological properties and physicochemical features [101]. In particular, type I collagen is the major component of the extracellular matrix (ECM) and popularly used for wound healing, promoting cell migration, and tissue regeneration [102]. But collagen type I has limitations for bioprinting: it is in a liquid state at low temperature and forms a fibrous structure when increasing the temperature. In addition, it is difficult to bioprint a 3D construct due to the slow gelation rate of collagen (> 30 min at $37\text{ }^{\circ}\text{C}$), and the deposited hydrogel remains in a liquid state for > 10 min [103]. Furthermore, the cell-laden collagen hydrogels suffer from an issue that the distribution of cells in the hydrogels is not homogeneous, because the gravity pulls down the cells before completing the gelation [35].

2.2.5 Methylcellulose

Methylcellulose (MC) is a chemically modified cellulose. It is a water-soluble polymer because of substitution of some hydroxyl groups in the chains of cellulose with some hydrophobic groups [87]. The gelation mechanism of MC goes through two stages. The first stage is hydrophobic interaction between highly methylated

glucose zones. The second stage is a phase separation process to form a turbid gel, which occurs at high temperatures $> 60\text{ }^{\circ}\text{C}$ [104]. The highly viscous MC can be used as a printing material [87], which demonstrates a good shape fidelity and stackability [78]. For instance, Schütz et al. [78] prepared an alginate/MC blend hydrogel for bioprinting. In their study, the cell-laden 3D structure with a high resolution, a good shape fidelity and the clinically relevant dimensions (~ 50 layers, the diameter of the extrusion nozzle $\sim 250\text{ }\mu\text{m}$) can be obtained.

2.2.6 Carrageenan

Carrageenans are a family of linear sulfated polysaccharides, which are extracted from the red seaweeds. They consist of repeating sequences of β -D-galactose and α -D-galactose. Based on the number of ester sulfate groups and their positions on the repeating galactose, carrageenans can be classified into three groups: kappa-, iota-, and lambda-carrageenan [105]. In the presence of cations, kappa- and lambda-carrageenans can form the reversible hydrogels through the transition from random-coils to double helices [106]. The kappa-carrageenan hydrogels are less deformable and stronger than the lambda-carrageenan hydrogels. The gelation of kappa-carrageenan goes through two steps: the coil-helix transition and aggregation of double helices [107]. The gelation of kappa-carrageenan is determined by concentration of kappa-carrageenan, temperature, and type and content of metal salts [108]. The degradation of carrageenan hydrogels is similar to alginate hydrogels,

which is driven by exchanging of ions with the surrounding medium [109]. To improve mechanical properties of carrageenan hydrogels, Liu et al., [110] prepared the stretchable kappa-carrageenan/polyacrylamide double network hydrogels. But as mentioned previously, the double network hydrogels may be unfavorable to cells due to the incorporation of a chemical network [9, 111].

2.2.7 Agarose

Agarose is a naturally-derived polysaccharide, which can be obtained from the cell walls of red algae [105]. It is a thermo-responsive hydrogel that has been used in the extrusion printing system at low concentrations (1%-5% w/v) [87, 112]. An agarose aqueous solution may undergo gradual gelation at low temperatures, but starts to liquefy when temperature is within a range of 20-70 °C [113, 114]. The gelation of agarose is achieved by three steps, which are namely initiation, nucleation, and pseudo-equilibrium [114]. Low cell adhesion and spreading indicate that agarose is not the suitable material for cell culturing [115]. But, it can serve as a sacrificial material to build a mold or pattern [87]. The gelation at low temperatures makes agarose difficult to be directly used as a printing ink.

2.3 Specific considerations for 3D bioprinting

Based on the review of currently used hydrogels for bioprinting, lacking of suitable hydrogels that are designed specifically for bioprinting is regarded as a major challenge in the field of bioprinting [116]. But the design criteria for successfully 3D

printing a construct for bio-application should be made clear. In this section, the specific considerations of the important properties of a candidate bioink and the generated constructs are summarized and discussed.

2.3.1 Rheological considerations

The suitability of a candidate hydrogel for bioprinting mainly depends on its physicochemical properties. Rheological properties of a hydrogel is the major physicochemical properties to determine its printability [4]. Rheology is the study of flow and deformation of a matter under the condition of an external force, which is highly relevant to an extrusion-based 3D bioprinting process.

A number of investigations have been carried out to evaluate the printability of hydrogels through rheological experiments. For example, Chung et al., [77] reported that the mixture of alginate and gelatin exhibited a better gel-like behavior when the printing was conducted at low temperatures, comparing to the performance of alginate or gelatin hydrogels at the room temperature. The gelation temperature for this mixture used in their study was 11 °C, which was determined by a rheological temperature-sweep measurement. Jia et al. found that for a piston driven system, the optimal range of the kinematic viscosity is from 400 to 3000 mm²/s [12]. Murphy et al., [7] claimed that a bioink with viscosity ranging from 30 to 6 × 10⁷ mPa·s was suitable for an extrusion based printer. Blaeser et al.,[23] pointed out the shear stress generated during the 3D bioprinting process should be considered when optimizing

the printing resolution and the cell viability. Moreover, many factors can determine the shear stress exerted on the hydrogels and the embedded cells, such as the size of the nozzle and the pressure utilized for extruding the hydrogels [23].

Although all the previous works have demonstrated that rheological properties of a candidate hydrogel are important in controlling the printability of a hydrogel, the effect of 3D printing process on the rheological behavior of a candidate hydrogel and the resultant quality of printing for a 3D structure are still not clear. The importance of rheological considerations for 3D printing is underestimated [4]. Here, the important rheological parameters that may have significant influence on the printability of a hydrogel were summarized.

2.3.1.1 Viscosity

Viscosity is the resistance of a fluid to flow under a stress. The viscosity of a hydrogel is predominantly determined by its concentration, molecular weight and temperature. A hydrogel must have sufficient viscosity to maintain the designed shape of a printed construct. But it was reported that hydrogels with high concentrations could restrict the proliferation of cells [7, 117, 118]. Thus, it is logical to use a hydrogel with low concentrations but high viscosity [4] to maintain the shape of a 3D printed structure.

2.3.1.2 Shear stress

Shear stress is an inevitable factor in any mechanical dispensing process. The

distribution of velocity and shear stress across the cross-section of a nozzle are illustrated in Figure 2.8, where the shear stress is zero when the hydrogel is in a static state (before printing). But during an extrusion process, a shear stress is applied to the hydrogel. The printing parameters, such as printing pressure, diameter of the nozzle, and the viscosity of the bioink will determine the shear stress exerted on the bioink and the embedded cells [23, 30, 119].

Figure 2.8 Schematic illustration for the distribution of velocity (u) and shear stress (τ) of the cells-laden hydrogels within a nozzle [23].

Shear stress plays a pivotal role in cell biology [23, 120, 121]. Blaeser et al.,[23] found that the viability of cells can be immediately affected by short-time exposure of the cell-laden hydrogels to high shear stresses, which were generated during the extrusion process. In their study, the L929 mouse fibroblasts survived by 91% and 76%, when the shear stress ranged from 5 to 10 kPa and >10 kPa, respectively. But at

a low level of shear stress (<5 kPa), the viability of cells could reach 96% (Figure 2.9 (a)). Figure 2.9 (b) demonstrated the relationship between the viability of cells and the applied shear stress[119].

On the other hand, when fine nozzles are generally used for 3D printing to obtain a 3D construct with a high resolution, shear stress also increases with decreasing the diameter of a nozzle.

Figure 2.9 (a) Effect of shear stress on viability of cells after printing. All the data were classified into three groups (<5 kPa, 5-10 kPa, and >10 kPa). The microscopic images on the right side showed the live (stained in green)/dead (stained in red) cells after printing [23]. (b) Percentage of survived cells vs maximum shear stress [119].

2.3.1.3 Shear thinning

Shear thinning refers to the non-Newtonian behavior of a fluid where the viscosity decreases with increasing shear rate [122, 123]. This phenomenon is caused by shear stress. Figure 2.10 (a) illustrates the shear thinning behavior of an alginate hydrogel when it is extruded from a syringe to a narrow nozzle. During extrusion the hydrogel is under shearing by which the physical crosslinks are broken and the polymer chains are aligned, which results in a decreased extent of entanglements and then reduction of its viscosity. During a 3D printing process, the shear thinning behavior implies a reducing viscosity of a hydrogel flowing within a fine nozzle. Therefore, viscous and thixotropic hydrogels are able to be easily extruded out through a narrow nozzle to protect the cells against the shear stress.

Most polymeric physical hydrogels exhibit a shear thinning behavior, such as sodium alginate [4, 23, 124-126]. Many groups have studied the rheological properties of alginate hydrogels with various concentrations [79, 126]. According to Basim et al., [126] the viscosity of the tested alginate hydrogels decreased with increasing shear rate, when the shear rate was varied over a range of 1-1000 s⁻¹ (Figure 2.10 (b)).

Figure 2.10 (a) Schematic illustration of the shear thinning behavior of GelMA/gellan gum hydrogels. The insert drawings demonstrate the shear thinning (ii), and recovery (iii) behavior of the hydrogels. Note: GelMA chains in red, gellan gum chains in white [4]. (b) Viscosity of alginate hydrogels at various concentrations [126].

2.3.1.4 Thixotropic property

After being extruded, the physically crosslinked network of a hydrogel, which is broken by shear stress (see Figure 2.10 (a-ii)), should be able to self-recover [4] (see Figure 2.10 (a-iii)). It is ideal that the viscosity of a hydrogel could sharply decrease when a shear rate is applied, but the viscosity can recover quickly after the shear rate

is removed. The extruded hydrogel filament should have sufficient mechanical strength to maintain its shape after printing. Therefore, a very important property for a suitable hydrogel for an extrusion-based printer is the thixotropic property, which should be considered when evaluating the printability of a hydrogel for bioprinting. Examples for thixotropic materials include thixotropic paints, and silk nanofibril-based hydrogels [127]. However, the methods for evaluating the recovering ability of a candidate hydrogel under shearing is still not reported in the literature.

2.3.2 Interfacial bonding

There are defects between layers in a 3D layered structure fabricated using a layer-by-layer printing process. The interfacial defects may lead to low stackability, and mechanically weak 3D constructs. However, only a few researchers have reported this issue. Some studies include a lap-shear test could be used to evaluate the interfacial bonding of a multilayered hydrogel structure [20, 21]. Meanwhile, the interfacial failure pattern is an indication of adhesion at the interface between two layers of a layered hydrogel construct [20, 21].

2.3.3 3D structures

Bioprinting a 3D construct with a high shape fidelity and a high vertical thickness is desired. Shape fidelity of a printed hydrogel is crucial for an extruded filament to maintain its shape and then support the subsequently printed structure, such as pores and channels without collapse. He et al.,[49] systemically studied the printability of

alginate/gelatin hydrogel (containing 2.5% alginate and 8% gelatin) hydrogels from printing lines to print 3D structures. The experiments with one-dimensional printing allowed them to find the optimized parameters, such as pressure and feed rate for printing. For sharp angle printing, an overlapping problem might be generated, as shown in Figure 2.11 (a1). This problem could cause each printed layer to have an uneven height, which may further result in a failure of printing after several layers are printed. The authors suggested two ways to avoid the overlapping issue in sharp angle printing [130]. One was to avoid printing patterns with sharp angles, while the other was to reduce the extrusion speed or increase the nozzle moving speed in the overlapping area. A printed angle with overlapping is shown in Figure 2.11 (a2), while a uniform shape can be obtained when utilizing two times nozzle moving speeds in the overlapping area (see Figure 2.11 (a3)).

For a lattice structure, the diffusion effect should also be considered when designing a pattern (see Figure 2.11 (b)). The grid structures with different line distances (D_L) were printed and compared [130]. The diffusion between two adjacent lines could even cause overlapping when D_L was 1 mm. But diffusion and fusion could be mitigated when D_L was 4 mm (see Figure 2.11 (c)).

Figure 2.11 Printability evaluation for alginate/ gelatin hydrogels. (a) Sharp angle printing. (a1) Schematic of overlapping in sharp angle printing. (a2) Result of utilizing a uniform nozzle moving speed in the overlapping area. (a3) Result of utilizing two moving speeds of nozzle in the overlapping area. (b) Schematic illustration of the directional diffusion of the printed lattice. (c) Printed lattice with the different D_L [49].

To date, most of the reported 3D constructs were relatively simple (e.g. rectangular prism) and with small thickness, which were suitable for in vitro tests or a small scale of animal in vivo tests [128]. For example, Yong et al.,[49] used the alginate/gelatin blend hydrogel (containing 2.5% alginate and 8% gelatin) to print a pre-designed

pattern as shown in Figure 2.12 (a). Although the printed construct was further solidified in a CaCl_2 bath after printing, the diffusion can still be observed (Figure 2.12 (b)). Moreover, the pore in the center cannot accurately maintain its originally designed shape, which was related with a weak stacking ability and a poor shape fidelity. Thus, a hydrogel with good shape fidelity and stacking ability is urgently needed for fabrication of relatively thick and robust structures [129].

Figure 2.12 Shape fidelity of the printed alginate/gelatin hydrogel (containing 2.5% alginate and 8% gelatin) constructs. (a) The pre-designed model of a 3D structure. (b) Images for the printed 30 layers structure [49].

2.3.4 Cell viability

Cell viability in a hydrogel is influenced by the type and concentration of the hydrogel, temperature, and culturing time [103]. Fedorovich et al., [115] compared the cell viability within various hydrogels over time, which included alginate (2% concentration), agarose (1% concentration), and Lutrol F 127 (25% concentration). Figure 2.13 illustrates that there was no significant difference in cell viability (bone marrow stromal cells) among different hydrogels, after incubation for 5 hours. But

after day one, the cell viability in Lutrol F 127 sharply dropped to 20%. After culturing for 7 days, cell viability in alginate can maintain ~ 90%. In contrast, the cell viability in agarose was only ~70%, and there were no viable cells in Lutrol F 127 to be detected.

Figure 2.13 The viability of cells in different hydrogels, which was examined at 5 hours, day 1, day 3, day 5 and day 7 [115].

Concentration of a hydrogel is also one of the factors affecting the cell viability. Ouyang et al.,[130] reported that a blend contained 1% alginate and 5% gelatin showed a cell viability of almost 100%. But in a blend of 1% alginate and 10% gelatin, the cell viability dropped to 70% at six hours after printing. Yu et al.,[131] found that a 2% alginate hydrogel maintained a 90% cell viability, but the alginate hydrogel with a higher concentration (6%) had a lower viability (35%). There are two reasons to explain this result. Firstly, cells generally survive in porous networks with cell binding domains [4] to facilitate cell spreading. A highly viscous hydrogel with a reduced porosity could prevent cells' spreading and migration [11, 132, 133]. Secondly, highly viscous hydrogels are usually utilized for bioprinting of constructs with a good shape

fidelity. But during a 3D printing process, highly viscous hydrogels may have to be extruded using high shear stresses that may affect cells laden in the hydrogels. The relationship between cell viability and shear stress has also been discussed in the previous section 2.3.1.2 (see Figure 2.9).

2.3.5 Degradation rate

Degradation rate of a 3D printed construct depends on the composition of bioink (for example, type of the selected hydrogel), concentration, temperature, and cell culture media. The degradation rate of a printed structure significantly affects its application. The printed scaffolds or cell-laden constructs must be stable under an in vitro culture condition or an in vivo environment within a desired time according to their applications [134]. It is because that hydrogels should provide cells with a 3D structural support until they can build their own extracellular matrix (ECM) proteins [7]. It is ideal that the degradation rate of a printed construct matches with the ability of cells to replace the printed structure with their own ECM proteins. Thus, the bioink for printing a 3D construct should have a suitable degradation rate, as it needs to keep the integrity of a desired structure until the tissue regeneration is almost completed. However, some hydrogels gradually lose their mechanical properties during culturing in a short time. As mentioned previously (Section 3.1), the mechanical strength of the alginate hydrogel was quickly lost (40% lost within 9 days) during in vitro culture. Thus, to further improve the integrity of a printed structure, the dispensed bioinks

were usually cross-linked either simultaneously during printing or after printing [43, 45, 135, 136]. For example, Tabriz and his group [42] fabricated a vascular structure using alginate hydrogel, and the printed structure started to break down in the culture medium from day 2. After BaCl_2 solutions with different concentrations were used as the post-crosslinking agent, the integrity of the printed structure in the culture medium was improved to 7 days. Meanwhile, the remaining scaffold after degradation may inhibit the tissue generation rather than promoting it, if the remaining scaffold stays for a longer period than necessary [137]. For this reason, a hydrogel with a suitable degradability should be carefully selected for diverse tissues types. For example, the scaffold used for skin's TE doesn't need to stay longer than one month [137]. Moreover, the degradation products should be nontoxic [7].

Conditions, including temperature, for use of 3D printed hydrogel constructs are another factor that needs to be considered. Naturally derived hydrogels, including collagen undergo enzymatic hydrolysis, which will result in a reduced mechanical strength [137]. Thermosensitive hydrogels are sensitive to temperature, which may lose their shape when the environment temperature changes. A typical example of thermosensitive hydrogels is gelatin. It inherits the superior performance of collagen to promote cell adhesion [138], but it dissolves as a colloidal sol at a cell culture temperature of 37 °C in a cell culture medium [88]. Based on these reasons, a hydrogel and its resultant 3D structure with a suitable degradation rate should be carefully selected.

2.4 Summary

Current 3D bio-fabrication technologies allow us to design and print constructs through layer-by-layer stacking of hydrogels and cells. The most prominent bioprinting technologies (extrusion printing, inkjet printing and laser-assisted bioprinting) that are utilized for 3D bioprinting were presented and compared. Each technology has its own advantages and limitations. Among all of them, extrusion printing is the only technology that can be used to fabricate clinically relevant size constructs. After that, most of the currently used hydrogels for bioprinting were described, and the shortcomings of each hydrogel for bioprinting were discussed. Lacking suitable bioink for bioprinting of thicker 3D structures is hampering the progress of bioprinting for fundamental research and clinical applications. This issue may be partially due to the current lack of systematic studies that focus on the characterization of the potential bioinks from a rheological point of view. Finally, the specific considerations of the important properties of bioinks and the generated 3D constructs were highlighted.

To successfully obtain a 3D construct for bio-application, the specific considerations for a candidate hydrogel and the generated structure, can be classified into two groups: during printing, and after printing. During a printing process, the candidate hydrogel should exhibit a shear-thinning behavior, which implies a decreased viscosity of the hydrogel within a narrow nozzle. Thus, a viscous hydrogel

could be easily extruded out through a fine nozzle. Meanwhile, shear stress is an inevitable factor in an extrusion process, which will affect the cell viability. Thus, the value of shear stress should be controlled. After printing, i) the hydrogel should be highly thixotropic, which can provide the extruded filament with sufficient mechanical strength to maintain its shape and then support the subsequently printed layers. However, a method for estimating the recovery property of a hydrogel has not been reported in the literature. ii) There are layer defects in the 3D printed constructs due to the layer-by-layer printing process. Therefore, the interfacial properties between the printed layers should be examined. However, this issue is rarely reported. iii) The hydrogel for printing should be able to generate a 3D complex construct with a high stackability and a high shape fidelity. iv) The printed structure should have an ability to promote cell viability, growth and proliferation. v) The bioprinted construct is degradable, but the structure must be stable under in vitro culture or in vivo environments over a desired time according to its applications.

Among all the considerations, interfacial bonding is one of the important considerations for successfully obtaining a 3D structure. But it is rarely mentioned in the literature. The importance of rheological considerations for 3D printing should be emphasized. The rheological behaviors of a candidate hydrogel before, during, and after printing should be understood clearly. Thus, simulating the rheological properties of hydrogels during a printing process could help us to find the relationship between printability and rheological behavior of a hydrogel. It is hoped that the above-

mentioned specific considerations for 3D printable hydrogels and their 3D printed constructs could help the researchers in selecting suitable hydrogels for bioprinting.

Chapter 3 Printability of a Model Hydrogel for the Extrusion-based 3D Printing

In this chapter, the objective is to report a novel approach for predicting the printability of a model hydrogel through simulating its rheological properties before, during, and after the 3D printing process.

3.1 Experimental design

Hydrogels prepared from natural polymers such as alginate (Alg) [12], gelatin [139], collagen [15], chitosan [18], were successfully used for bioprinting. But these natural hydrogels have limitations for bioprinting, which has been discussed in Chapter 2. Thus, there is an urgent need to develop a novel bioink for bioprinting a 3D construct with good printability. Rheology is the study of the flow of materials under application of an external force, which is highly relevant to an extrusion-based bioprinting process. But, in the literature, it is still not clearly reported what is the relationship between rheological properties of hydrogel and its 3D printability. Thus, this chapter aims to find out the desired rheological properties of a suitable hydrogel for 3D printing and to develop a novel approach to evaluate the printability of a candidate hydrogel for 3D printing.

A printable hydrogel needs to be optimized to have low viscosity during printing and sufficient mechanical strength after printing. Therefore, it is ideal for a printable hydrogel to have a thixotropic property and a fast recovery ability. For a non-

Newtonian fluid, viscosity is a function of shear rate in a printing syringe. To find the relationship between piston speed and shear rate for an extrusion-based printing process is fundamentally important. It is also important to know whether the breakdown of crosslinks by shearing is reversible after removing the shear force. Thus, before studying the thixotropic property of a candidate hydrogel, the value of shear rate which is generated during the printing process should be estimated.

In this chapter, firstly, an equation will be deduced for estimating the value of shear rate exerted on the hydrogel during the extrusion process. After that, alginate (Alg) is selected as a model hydrogel to test our rheological method for simulation of the rheological properties of the hydrogel during the 3D printing process. Rheological studies are first conducted for the Alg hydrogel, which help us to determine the value of the shear rate exerted on the Alg hydrogel during the extrusion process. Then, the appropriate shear rates will be applied on the Alg hydrogel to simulate its thixotropic properties before, during and after printing. The observed recovery ability for a hydrogel (e.g. recovery percentage of viscosity, and recovery time) is an indication of its printability. Furthermore, graphene oxide (GO) is also added to modify the rheological properties and 3D printability of the Alg based hydrogels.

3.2 Materials and methods

3.2.1 Materials and sample preparations

Sodium Alg was purchased from Sigma-Aldrich, Singapore. According to the

supplier, the molecular weight of the Alg ranged from 100,000 to 150,000 g/mol and the G block content was 50-60%. Calcium chloride with 99% ACS grade was obtained from Sigma-Aldrich, Singapore. Graphene oxide (GO) was a product of XF NANO (Nanjing, China). All materials were used without further purification.

Aqueous solutions of Alg with various concentrations (2, 4, 6, 8 and 10 wt%) were prepared using deionized water (DI water) from a Millipore water purifier. Then, calcium chloride solutions with various molar concentrations were added to each solution of Alg to obtain Alg hydrogels with various CaCl_2 contents. To study the effect of GO on Alg hydrogels, Alg composite hydrogels filled with various GO concentrations were prepared as follows. First, the suspensions with various GO contents were produced by ultrasonic treatment using DI water. After that, a certain amount of Alg powder was added into the suspension of GO under magnetic stirring. Finally, Alg composite hydrogels were prepared by adding a certain amount of calcium chloride solution into the solution of GO/Alg under magnetic stirring. The Alg concentration in the composite hydrogels was fixed at 10 wt% and a CaCl_2 concentration of 25 mM/L was also kept constant, while GO was added into the mixture with various contents (0.05, 0.15 and 0.25 wt%, which was based on the weight of total DI water). The prepared samples were labelled with GO_a/Alg_b, where a and b were the weight fractions of GO and Alg, respectively.

3.2.2 Rheological evaluation of the printability of hydrogels

The rheological properties of the Alg hydrogels were measured by using a rotational rheometer (DHR, TA Instruments, USA). A 40mm parallel plate with a measurement gap of 0.55mm was used. First of all, strain sweeps in the range of 0.1 – 100 % at frequencies of 0.1 – 2 Hz were carried out to determine the linear viscoelastic range of the samples. The following three rheological experiments at room temperature were adopted for exploring rheological properties of samples: (1) frequency sweep tests over an angular frequency range of 0.01-100 rad/s at a constant strain of 2 %; (2) steady-state flow tests in a range of shear rate 0.5 – 500 s⁻¹; and (3) recovery tests under an estimated shear rate.

3.2.3 3D printing

A piston driven extrusion-based printer (see Figure 3.1) was employed in this study. The printing system consists of two parts: a high precision displacement pump (TechnoDigm, PDP 1000, Singapore) and a desktop xyz motor (TechnoDigm, DR3331T-EX, Singapore). The printing head was mounted on the printing system to print along the pre-designed tracks with an adjustable speed (15 mm/s used in this study). The printing head consists of a piston, a syringe and a changeable nozzle. The displacement pump drives the piston with a controllable speed (0.009mm/s) to extrude a hydrogel from the syringe on a glass slide. The 3D structures were fabricated at room temperature. Firstly, a pattern was pre-designed on the 3D printing system to

define the extrusion route for the hydrogel. Secondly, the hydrogel was loaded into the syringe and then the syringe was installed on the dispensing unit. Under the action of the piston at a speed, the hydrogel loaded in the syringe was extruded through a 0.25 mm nozzle while the dispenser was moving at a defined speed. Once the first layer was formed, the nozzle was lifted up and then continued to print the second layer. Subsequent layers were printed layer by layer in the vertical axis.

Figure 3.1 Image of extrusion-based 3D printer driven by mechanical force

3.3 Results and discussion

3.3.1 Sol-gel transition

Alg is able to form a gel in the presence of CaCl_2 . Figure 3.2 illustrates the dependence of storage modulus G' and loss modules G'' on angular frequency ω for the aqueous solution of 2 wt% Alg containing various CaCl_2 concentrations.

Figure 3.2 Dependence of G' and G'' on angular frequency for 2 wt% Alg hydrogels with various contents of CaCl_2 . (a) Dependence of G' on angular frequency; (b) Dependence of G'' on angular frequency.

At low CaCl_2 concentrations, such as 2.5, 3.75 and 5 mM/L, G'' was larger than G' in the low frequency region. These correspond to the viscoelastic properties of a polymer fluid without entanglements. After adding 6.25 mM/L of CaCl_2 into the Alg

solution, both the G' and G'' became much higher than those at 5 mM/L of CaCl_2 in the whole frequency region. It is noted that G' is larger than G'' , showing a characteristic of a solid-like material. There is an obvious gap between the curves for 5 mM/L and 6.25 mM/L. The large increase from the G' curve at 5 mM/L of CaCl_2 to that at 6.25 mM/L of CaCl_2 implies that the gelation of Alg solution takes place at a concentration of calcium ions between 5 mM/L and 6.25 mM/L.

A method was developed by Winter and Chambon [140] to determine the exact critical gel concentration. The main feature of this method is the scaling law at the gel point: both $G'(\omega)$ and $G''(\omega)$ are proportional to ω^n at sufficiently low frequencies, ω , where n is the scaling index ($0 < n < 1$). The definition of the gel point by this power law is excellent because the gelation variable will lose its dependency of frequency at the gel point. Many works have shown that this method is reliable and valid for determination of the gel point for various polymer gels with different gelation mechanisms [141-143]. Figure 3.3 (a) shows the application of the Winter-Chambon method to the solution of 2 wt% Alg within the sol-gel transition region. All curves passed through the common point at a certain CaCl_2 concentration of 5.73 mM/L, and this point was defined as the critical gel concentration (C_g) for the solution of 2 wt% Alg. The similar multi-frequency curves of tangent delta versus CaCl_2 concentration were also obtained for other Alg solutions, and the critical gel concentrations obtained are shown in Figure 3.3 (b). It is observed that C_g increases linearly with increasing Alg concentration, indicating that more calcium ions are required to form cross-link

with Alg chains at a higher Alg concentration.

Figure 3.3 (a) Dependence of $\tan \delta$ on CaCl_2 concentration for 2 wt% Alg hydrogels at different angular frequencies in rad/s as indicated in the inset, and (b) relationship of the critical gel concentration of CaCl_2 (C_g) with Alg concentration.

3.3.2 Rheological evaluation of the printability of hydrogels

3.3.2.1 Determination of shear rate

The 3D printing head consists of a piston, a syringe and a nozzle. The syringe and nozzle have different inner diameters. Before printing, the hydrogels are loaded into the syringe firstly and then it is extruded to the nozzle under the pushing action of a piston. On the basis of the fluid mechanics [123], the viscosity of a non-Newtonian fluid is a function of shear rate. At a constant volume flow rate, the linear flow rate will change due to the change in the cross-sectional area from the syringe to the nozzle. All these will lead to a change in the shear rate. Thus, the shear rate is an important factor for understanding the behavior of hydrogels during the 3D printing process.

Consider a laminar and steady flow of a time-independent and incompressible fluid in a circular pipe of radius (R), as shown in Figure 3.4 (a). Since there is no angular velocity, the force balance in the z direction on a fluid element situated at a radius r ($0 < r < R$) can be written as

$$p (\pi r^2) - (p + \Delta p) \pi r^2 = 2\tau \pi r L \quad (3.1)$$

$$\tau = \frac{-\Delta p}{2L} r \quad (3.2)$$

where p is the pressure, τ is the shear stress on the surface of the cylindrical element, L is the length of the element, and Δp is the pressure drop. Equation (3.2) shows the shear stress distribution across the cross-section of pipe, the shear stress being zero at the axis of the pipe (Figure 3.4 (b)). Note that equation (3.2) is applicable to both

turbulent and laminar flows of any fluid since it is based on a simple force balance and also no assumption has been made [123].

Figure 3.4 (a) Flow through a pipe, and (b) Stress and velocity distribution of non-Newtonian flow in a pipe with a radius R .

For a power-law fluid in a pipe, shear stress is a function of shear rate as follows [144]:

$$\tau = m(\dot{\gamma})^n \quad (3.3)$$

where n and m , are the power-law index and power-law consistency coefficient, respectively. $\dot{\gamma}$, is the shear rate. Thus, the viscosity for the power-law fluid can be described by

$$\eta = m(\dot{\gamma})^{n-1} \quad (3.4)$$

The shear rate can be written as

$$\dot{\gamma} = \frac{du}{dr} = \frac{-\Delta p}{2\eta L} r \quad (3.5)$$

where u is the flow velocity at r . Integrating the equation, the velocity in the pipe could be described as

$$u = \frac{-\Delta p}{4\eta L} (R^2 - r^2) \quad (3.6)$$

Rheological study was conducted on the Alg based hydrogels with different concentrations of Alg using a rotational plate rheometer. Based on the power-law

model and experimental data, the constants m and n for each sample can be obtained through curve fitting. These two parameters were used to deduce the shear rate that the hydrogel experienced during the printing process.

Assuming that in the syringe there is a uniform flow rate (V), the volumetric flow rate (Q) of a non-Newtonian fluid can be write as follows [123],

$$Q = \pi R^2 V = \pi \left(\frac{n}{3n+1} \right) \left(\frac{-\Delta p}{2mL} \right)^{\frac{1}{n}} R^{\left(\frac{3n+1}{n} \right)} \quad (3.7)$$

Then the pressure drop Δp can be expressed as follows

$$\Delta p = -2mL \left[\frac{V(3n+1)}{n} \right]^n \frac{1}{R^{n+1}} \quad (3.8)$$

The shear rate $\dot{\gamma}$ in the pipe can be described as follows

$$\dot{\gamma} = \left[\frac{V(3n+1)}{n} \right] \left(\frac{r}{R^{n+1}} \right)^{\frac{1}{n}} \quad (3.9)$$

Assuming that the volume of hydrogels does not change before and after printing,

$$V_2 = V_1 \left(\frac{D_1}{D_2} \right)^2 \quad (3.10)$$

where D_1 is the inner diameter of the syringe, V_1 is the moving speed of the piston, D_2 is the inner diameter of the nozzle, and V_2 is the speed of extruded hydrogel in the nozzle,

From equation (3.9), the shear rate $\dot{\gamma}$ in the syringe can be calculated from the following equation

$$\dot{\gamma}_1 = \left[\frac{V_1(3n+1)}{n} \right] \left(\frac{r}{R_1^{n+1}} \right)^{\frac{1}{n}} \quad (3.11)$$

Finally, the shear rate $\dot{\gamma}$ in the nozzle can be calculated from the following

equation

$$\dot{\gamma}_2 = \left[\frac{V_2(3n + 1)}{n} \right] \left(\frac{r}{R_2^{n+1}} \right)^{\frac{1}{n}} \quad (3.12)$$

3.3.2.2 Evaluation of the printability of hydrogels

The aim of this chapter is to evaluate the printability of a model hydrogel through rheological measurement. Figure 3.5 (a) shows the flow curves over a range of shear rates ($0.5 - 500 \text{ s}^{-1}$) for Alg hydrogels at a fixed CaCl_2 content of 25 mM/L. A shear thinning behavior was observed for all samples. The viscosity of the hydrogels increased with increasing Alg concentration but decreased with increasing shear rate. This is the most common behavior of a non-Newtonian fluid [123]. Specially, the influence of shear rate on viscosity for the 2 wt% Alg solution was more significant than that for the 10 wt% Alg solutions.

An ideal printable hydrogel should be highly thixotropic, which means that viscosity of the hydrogel become low quickly when applying a shear force. But the viscosity recovers quickly after the shear force is removed. It is also important to know how the crosslinks of the hydrogel can recover before the next layer starts to be printed. Thixotropic properties and recovery times are investigated by applying a steady shear rate on the hydrogels. Thus, the value of shear rate generated during a 3D printing process should be estimated before conducting the rheological test. As discussed in the previous section 3.3.2.1, the shear rate can be calculated using equation (3.12). Firstly, rheological measurements were performed on samples. Based on the power-

law model, the constants m and n for each sample can be obtained through curve fitting (see Table 3.1).

Figure 3.5 (a) Viscosity of Alg hydrogels as a function of shear rate at room temperature, and (b) effect of various Alg concentrations on the recovery behavior of Alg hydrogels at a fixed CaCl_2 concentration of 25 mM/L.

Table 3.1 m , n for each hydrogel and the maximum shear rate exerted on the hydrogel in a nozzle

Samples	m	n	Shear rate (s^{-1})
Alg2	12.5	0.147	170.5
Alg4	25.4	0.288	112.8
Alg6	64.8	0.329	105.2
Alg8	115.3	0.390	97.0
Alg10	172.5	0.424	93.4
GO0.05/Alg10	223.9	0.399	96.0
GO0.15/Alg10	257.4	0.355	101.4
GO0.25/Alg10	360.2	0.305	109.5

The viscosity of 2 wt% Alg hydrogel was too low so that the printed structure collapsed quickly. Thus, this sample was not appropriate for 3D printing. For the other concentrations of hydrogels (see Table 3.1), the maximum shear rate in the nozzle for each sample was around $100 s^{-1}$ at the speed of 0.009 mm/s for pushing piston (V_1) used in this chapter, where the diameter of the syringe and the diameter of the nozzle were 3.88 mm and 0.25 mm, respectively. Therefore, each sample was applied under a shear rate of $100 s^{-1}$.

From Figure 3.5 (b), the whole test consists of three steps. At step I, a shear rate of $0.1 s^{-1}$ was applied for 60 seconds. This step simulated the initial state of a hydrogel before printing. At step II, the shear rate was increased to $100 s^{-1}$ and held for 10

seconds. This step simulated the condition for a hydrogel under a certain shear rate during the printing process. At step III, the shear rate was reduced to 0.1 s^{-1} and held for 60 seconds to simulate the final state of the hydrogel after printing. Figure 3.5 (b) shows the recovery behavior of Alg hydrogels. In the case of the Alg10 hydrogel at step I, the initial viscosity was $582 \text{ Pa}\cdot\text{s}$. Then the viscosity sharply decreased to $11.87 \text{ Pa}\cdot\text{s}$ when the shear rate increased to 100 s^{-1} . After removing the shear rate, the viscosity built up to $465 \text{ Pa}\cdot\text{s}$ in about 10 s, which was a 79.7 % recovery of the initial value. If a longer recovery time (20 s) was considered, the viscosity could recover to $484 \text{ Pa}\cdot\text{s}$ (83 % of the initial value). The hydrogel can recover its viscosity by 85.5 % of the initial value after 30s, but the viscosity could not recover further even with a longer recovery time. The reason for the viscosity of a hydrogel to recover after a period of rest is because the broken crosslinks caused by shearing need some time to rebuild. The recovery time decreased as the Alg concentration increased, but most of the tested samples could not recover in few seconds and they need more than 30 seconds to recover their viscosities to 83 % of the initial values.

From Table 3.1, several features can also be observed. As the concentration of Alg increased from 2 wt% to 10 wt%, the power law consistency coefficient (m) and power law index (n) also increased. This is explained through an increased number of polymer chains at a higher mass concentration. Thus, the viscosity increases as the polymer concentration increases [145]. Similarly, m and n gradually increase as the viscosity increases. For a shear-thinning fluid, n should be smaller than 1 [123]. All

the tested samples showed that the values of n were smaller than 1, indicating that all of them had the shear-thinning properties, as proved in Figure 3.5 (a). Furthermore, as the concentration of Alg increased from 2 wt% to 10 wt%, the extent of shear-thinning became gentler. This implies that an Alg hydrogel with a higher concentration shows a weaker shear-thinning behavior than the one with a lower Alg concentration, and the former has a bigger value of n . This phenomenon was also mentioned and discussed in the Chhabra and Richardson's book [123]. If the value of n can achieve one, the viscosity will be a constant and not dependent on shear rate. For a shear-thinning fluid ($0 < n < 1$), when the value of n approaches to 1, the fluid behaves similar to a Newtonian fluid.

3.3.3 Quality of printing for Alg hydrogel without GO

Printing a hydrogel into a 3D structure in the vertical direction is very challenging. The strength of the hydrogel must be high enough to withstand the weight of the entire structure. This is quite difficult for hydrogels as they are soft materials with high water content. Insufficient structural strength of the hydrogel base can result in the collapse of the structure in the vertical configuration. Thus, the viscosity and the mechanical strength of the hydrogel material should be relatively high in order to suffer from the compressive pressure generated from the upper layers of the printed structure.

In this chapter, the speed for pushing the piston was 0.009 mm/s (V_I), the inner diameter of the microneedle (i.e. the nozzle) was 0.25 mm. To study the stability and

quality of printing, the images of the freshly printed constructs were recorded. Figure 3.6 shows the effect of Alg concentration on the printed hydrogel structures at a fixed CaCl_2 concentration of 25 mM/L.

Figure 3.6 Effect of Alg concentration on the printed structures of Alg hydrogels with a fixed CaCl_2 concentration of 25 mM/L. (a) The images of 9-layer grids printed with different concentrations of Alg, and (b) Effect of Alg concentration on the filament width.

The printed structures shown in Figure 3.6 (a) had 9 layers. It is obvious that for the structures printed with a higher Alg concentration, the printed filaments were

thinner. If the filament width was defined as d , the value of d decreased with increasing Alg concentration, as shown in Figure 3.6 (b). This is because that the hydrogel with a higher concentration of Alg is stronger and not easy to collapse when compared to the hydrogel with a lower Alg concentration. Thus, a smaller width implies a better printing quality of the hydrogel. The shape fidelity of the printed structures over an observation time were also investigated (see Figure 3.7).

Figure 3.7 Effect of aging time on the printed hydrogel structure for 2 wt% Alg hydrogels with a fixed CaCl_2 concentration of 25 mM/L. (a) The images of 9-layer grids printed observed at different ageing times and (b) Effect of ageing time on filament width.

Figure 3.7 (a) shows the images of the printed hydrogel structures at different ageing times. The relationship between the filament width and the ageing time for 2 wt% Alg hydrogels (containing a fixed CaCl_2 concentration of 25 mM/L), is illustrated in Figure 3.7 (b). It is obvious that the shape of the printed structures changed with time as the used hydrogel was soft and easy to collapse.

3.3.4 Quality of printing for Alg hydrogel with GO

Alg composite hydrogels filled with various GO contents were used to print 50-layers structures to study the effect of GO on the 3D printability of Alg hydrogels. From Figure 3.8 (a), it was observed that the hydrogels filled with GO also exhibit the shear thinning properties. GO can increase the viscosity of hydrogels, and the viscosity increased with increasing the content of GO. On the other hand, it was found that the hydrogels with various GO contents also exhibited the thixotropic properties, as shown in Figure 3.8 (b). Furthermore, the recovery rate for the GO0.25/Alg10 composite hydrogels does not show significant improvement compared to Alg10, as shown in Figure 3.8 (b). The initial viscosity for GO0.25/Alg10 was 1388 Pa·s. Then the viscosity sharply decreased to 13.66 Pa·s when the shear rate increased to 100 s^{-1} . After removing the shear rate, the viscosity built up to 1124 Pa·s (81 % recovery of the initial value) in about 30 s. Thus, the recovered viscosity for GO/ Alg10 was still much higher than that of Alg10 as the initial viscosity for Alg/GO was higher than that of Alg10.

Figure 3.8 Effect of GO contents on (a) shear thinning behavior, and (b) thixotropic property of 10 wt% Alg hydrogels.

Figure 3.9 (a) shows effect of GO on the morphologies of the printed structures with 10 wt% Alg hydrogels. The higher content of GO produced a structure with a thinner width. GO is essentially an atomic sheet with a large number of functional groups (e.g. hydroxyl, epoxide, and carbonyl groups) bound on the surface. After

adding GO into the Alg solution, the functional groups (such as -OH, -COOH) on GO interact with the groups (-OH) of Alg. Thus, the viscosities of the composited hydrogels could be significantly improved due to a large number of hydrogen bonds formed between GO and Alg.

Figure 3.9 The morphologies of the 50-layer structures printed with 10 wt% Alg hydrogels filled with various GO contents (a) without a recovery time, $t = 0$ second, and (b) with a recovery time, $t = 30$ seconds. The effect of GO content on the (c) width, and (d) height of the printed filament.

Furthermore, the traditional process of 3D printing is to continuously print a 3D structure layer-by-layer and there is no pause between the two layers. However, the continuous printing process without any pause between two layers seems unreasonable for the hydrogels used for an extrusion-based printer. It is because that the lost viscosity due to shear needs a period of time to be recovered. Here, a recovery time is defined, which is the duration from finishing print the current layer until the printer starts to print the new layer. Therefore, it would be necessary to find a reasonable recovery time that can improve the quality of printing. Based on the previous discussion in section 3.3.2.2, a recovery time of 30 s was set as the extruded hydrogels could recover most of its viscosity after 30 s and the viscosity cannot recover more significantly with longer recovery time. After that, the quality of printing of hydrogel was obviously improved when utilizing a recovery time, as proved by comparing the images of the structures printed with a recovery time ($t=30$ s, see Figure 3.9 (b)), and without a recovery time ($t=0$ s, see Figure 3.9 (a)).

Figures 3.9 (c) and (d) show the effect of GO content on the width and height of filaments for the structures fabricated without a recovery time and with a recovery time, respectively. It can be seen that the width of filament for a structure printed without a recovery time was bigger than that printed with a recovery time ($t = 30$ s). This is because the most viscosity of a hydrogel already recover after 30 s. Thus, the printing quality was better for those structures fabricated with a recovery time. At the same time, the filament height for the structure fabricated with a recovery time was

greater than that without a recovery time.

Figure 3.10 (a) Width, and (b) height of filaments (printed without a recovery time, $t=0$) as a function of aging time for Alg hydrogels filled with various GO contents.

From Figure 3.10 (a), it is observed that the value of width gradually increased with ageing time. Furthermore, as the content of GO increased from 0 to 0.25 wt%, the time effect became gentle. It is because that the hydrogel with a lower concentration of GO is softer and easier to spread when compared to the hydrogel with a higher GO content. Figure 3.10 (b) illustrates the relationship between height

and time with varying GO content. The heights of all structures decreased gradually with increasing ageing time due to the spreading effect. The height of a structure with more GO (GO0.25/Alg10) was always thicker than those structures with a lower GO content during the observation period (20 min).

Figure 3.11 shows the effects of ageing time on width and height of filaments for 10 wt% Alg hydrogels filled with various GO contents with the recovery time $t = 30$ s. From Figure 3.11 (a), it was observed that the width gradually increased as the ageing time increased. Compared to the recovery time $t = 0$ s (Figure 3.9 (a)), it was easy to find that the structures printed with the recovery time $t = 30$ s had a better quality. The recovery time also had effect on the height (Figure 3.11 (b)). The structures printed with a recovery time (Figure 3.11 (b)) showed the gentler decreased in height with ageing time than those structures fabricated without a recovery time. This is because the constructs printed with taking the recovery time are stronger, and thus the spread effect become less obvious for those printed with a recovery time. Therefore, taking a certain recovery time before printing next layer is a possible way to increase the quality of printing when using hydrogels with a slow recovery property (e.g. pure Alg hydrogels, GO-filled Alg hydrogels).

Figure 3.11 (a) Width, and (b) height of filaments (printed with a recovery time, $t = 30$ s), as a function of ageing time for Alg hydrogels filled with various GO contents.

3.4 Summary

In this chapter, a new approach was demonstrated to simulate the rheological behaviors of a non-Newtonian hydrogel during an extrusion-based 3D printing process. The shear rate in the printing nozzle could be estimated through a theoretical

analysis, and the viscosity versus shear rate profile. During an extrusion process, an ideal hydrogel should exhibit a shear-thinning behavior for easy extrusion through a narrow nozzle. The thixotropic property indicates how quickly and how much viscosity of a hydrogel can recover after printing, while the recovery time is the time given to the hydrogel for recovering its viscosity during a 3D printing process.

Alg hydrogel was selected as a model hydrogel and its rheological properties as well as 3D printability have been studied. The effects of CaCl_2 content and Alg concentration on the gelation properties of Alg in aqueous solution were also investigated. The gel point was determined using the Winter-Chambon method. It was found that the critical concentration of CaCl_2 at the gel point increased linearly with increasing Alg concentration, indicating that much more calcium ions are required to cross-link Alg chains into gel networks at a higher Alg concentration. The Alg/ CaCl_2 hydrogels exhibited a shear-thinning characteristic. However, the thixotropic properties of the Alg/ CaCl_2 hydrogels indicated that this hydrogel is not suitable for 3D printing because its poor recovery ability after printing. The printability of Alg hydrogel could be improved by adding a small amount of GO. The present study provides a simple and useful way to analyze the 3D printability of a hydrogel through a rheological point of view.

Chapter 4 3D Printing of Highly Thixotropic Alginate/Methylcellulose Hydrogel with Strong Interface Bonding

This chapter aims to report a strategy for printing constructs with strong interfacial bonding. The feasibility of utilizing a robust hydrogel blend and an interfacial improving agent was discussed.

4.1 Introduction

3D bioprinting technologies have significantly improved our capability to fabricate artificial tissues or organs through layer-by-layer stacking of biomaterials and cells [7, 146]. However, bioprinting of a 3D construct with great spatial control is still a challenge [147]. On the one hand, the printed constructs should also have sufficient mechanical strength to support the 3D structure without collapsing [148]. On the other hand, living cells must be deposited in the constructs while printing without seriously affecting the cells' viability and phenotype [5]. Moreover, there are often layer defects in 3D printed constructs due to the layer-by layer printing technology [20, 21].

The low viscosity hydrogels are generally mechanically weak and cannot maintain the shape of a printed structure. One of the strategies to obtain a shape-stable construct is by utilizing UV-cross-linkable hydrogels as the bioink. The low viscosity hydrogels, such as GelMA [89], are mechanically weak but become strong after

covalently crosslinked by exposure to UV light. However, there is a potential disadvantage of UV for cells [4, 42]. Bioinks with high viscosity can also be utilized in bioprinting [111, 149, 150]. Few studies have successfully demonstrated the possibility of printing of complex and tall constructs to mimic tissues or organs [151].

A good printability of highly viscous hydrogels with an extrusion-based bioprinter is associated with three main characteristics. First, the hydrogels should be highly thixotropic. Second, the hydrogels must have sufficient mechanical strength to support the subsequently printed structures. Third, the interfacial strength between hydrogel layers should be sufficiently strong to prevent delamination during and after printing. The resultant 3D shape fidelity of a 3D printed construct is a direct indication of the good printability.

Alg based hydrogels are popularly used for bioprinting. However, there is a printing height limit due to the poor stackability of Alg, which has been discussed in detail in Chapter 3. When Alg is mixed with another polymer, such as pectin [152] and chitosan [153], an Alg-based blend hydrogel with desired mechanical strength and printability may be obtained. Methylcellulose (MC) has been widely used as a viscosity-enhancing agent in food and pharmaceutical industries. As such, the addition of highly viscous MC could greatly enhance the viscosity of an Alg hydrogel [78].

In this chapter, a promising blend hydrogel of Alg/MC is presented. The rheological properties of the Alg/MC blend hydrogels are investigated as a function of the hydrogel composition. The shape fidelity and stackability of the optimized

Alg/MC hydrogels are evaluated. Furthermore, the interfacial properties between the printed layers are also examined, which are rarely reported in the literature [20, 21]. A strategy for improving the adhesion between the printed layers of layered construct is demonstrated.

4.2 Materials and methods

4.2.1 Materials and sample preparation

Sodium Alg with guluronic acid block (G block) content of 50-60%, methylcellulose (MC) (MW = ~88 kDa), calcium chloride (CaCl_2) and trisodium citrate (TSC) were purchased from Sigma-Aldrich, Singapore. The Hanks' balanced salt solution without calcium and magnesium (HBSS), fetal bovine serum (FBS), and antibiotic/antimycotic solution were obtained from ThermoFisher Scientific, Singapore. A high glucose Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS) were obtained from GE healthcare life sciences, Singapore.

To formulate Alg/MC blend hydrogels with various MC contents, a stock Alg hydrogel was first prepared. All the solutions were prepared with HBSS. The Alg hydrogel was prepared by adding a CaCl_2 solution (3 mg/ml) to an Alg solution (40 mg/mL) at a volume ratio of 1:3. The mixture was magnetically stirred overnight at room temperature to obtain a homogeneous hydrogel. Next, the Alg hydrogel was heated to ~ 80 °C to incorporate the MC. The MC powder was gradually dispersed

into the hot hydrogel at an Alg/MC ratio of 3:1, 3:3, and 3:9, respectively, where the Alg/MC ratio was based on the dry weights of Alg and MC. The mixture was thoroughly stirred until the MC powder was evenly dispersed while simultaneously allowing the mixture to gradually cool to room temperature. As soon as the mixture reached to room temperature, the MC powder began to hydrate and the viscosity of the mixture increased. But the full dissolution of MC was achieved by storing the mixture in a refrigerator at $\sim 4\text{ }^{\circ}\text{C}$ for at least 20 min and the Alg/MC blend hydrogel was then obtained.

The prepared blend hydrogels were named Alg3/MC1, Alg3/MC3 and Alg3/MC9, corresponding to the Alg/MC ratios of 3:1, 3:3 and 3:9 (wt/wt), respectively. The pure Alg hydrogel (contained 3 wt% Alg and named Alg3) served as a control. For comparison, MC1 (1 wt% MC), MC3 (3 wt% MC), and MC9 (9 wt% MC) were also prepared by gradually adding the MC powder into the hot HBSS solution.

4.2.2 Rheological measurement

To investigate the effect of MC on Alg hydrogels, the rheological properties of the Alg/MC blend hydrogels with various MC contents were measured using a plate rheometer (DHR, TA Instruments, USA) equipped with a 40 mm parallel plate and a 0.55 mm measurement gap. Two rheological tests at $25.0 \pm 0.1\text{ }^{\circ}\text{C}$ were adopted to explore the rheological properties of hydrogel samples: (1) steady-state flow tests; (2) recovery tests under a calculated shear rate simulating the extrusion process for 3D

printing.

4.2.2.1 Steady-state flow tests

To evaluate the viscosity and shear thinning properties of the hydrogels, steady-state flow tests of pure alginate hydrogel (Alg3), pure MC hydrogels (MC1, MC3, and MC9), and their blend hydrogels (Alg3/MC1, Alg3/MC3, and Alg3/MC9) were conducted at 25 °C over a range of shear rate of 0.5 – 1000 s⁻¹.

4.2.2.2 Determination of shear rate

In the nozzle, the shear rate $\dot{\gamma}$ exerted on a hydrogel at a radial position r ($0 < r < R$) [123], could be estimated by the deduced equation (3.9) as described in Chapter 3.

Here, the flow rate for the hydrogel within the nozzle should be calculated before utilizing equation (3.9). However, the information on the flow rate could not be directly obtained as the bioprinter (Biofactory bioprinter machine, RegenHU) used in this chapter is driven by pressure. The printing pressure utilized for each hydrogel had to be optimally adjusted. The inner diameter (I.D.) of the nozzle used was 0.25 mm (25 GA). The optimal pressure for extruding each hydrogel was the minimum pressure when a continuous filament could be deposited with a uniform filament diameter. The printed filaments were observed under an optical microscopy (OM, Zeiss Axio Vert. A1). The extrusion times, that is, the durations for the hydrogels (with a certain volume) to be fully extruded out from the nozzle under their respective

optimal printing pressures, were recorded for the calculation of the flow rate.

4.2.2.3 Characterization for thixotropic property

Thixotropic properties and recoverability of the hydrogels were examined. The rheological properties of hydrogels before (step I), during (step II), and after (step III) the printing process were simulated. At step I, a shear rate of 0.1 s^{-1} was applied for 60 seconds, which simulated the initial state of a hydrogel before printing. Step II simulated the sheared hydrogel during extrusion. The shear rate, which was calculated previously, was applied and hold for 5 seconds before moving to step III. At step III, the shear rate was reduced to 0.1 s^{-1} again and held for 60 seconds. This step simulated the final state of the hydrogel after printing.

4.2.3 Morphological characterization

The morphologies of Alg3, MC9, and Alg3/MC9 hydrogels were viewed under a scanning electron microscope (SEM, JEOL JSM-5600LV). The hydrogel samples were frozen in a freezer at $-30 \text{ }^{\circ}\text{C}$ for 24 hours, which were then freeze dried for 2 days. The top and cross-sectional surfaces were imaged separately under SEM, whereby the cross-sectional structures were obtained by fracturing the samples in liquid nitrogen.

4.2.4 Structural integrity of Alg3/MC9 sample

The structural integrity of the Alg3/MC9 hydrogel sample was observed in DI water at $37 \text{ }^{\circ}\text{C}$ for 30 days. Three cast cylindrical samples (15 mm in diameter and 8

mm in height) post-soaked in a CaCl_2 bath (40 mg/mL for 10 min) were tested. At definite time intervals, the samples were dabbed dry and weighed. The relative percentage of degradation (W_r) was calculated by $W_r = \left(\frac{W_1}{W_0}\right) \times 100\%$ [127, 154], where W_0 and W_1 were the weights of a hydrogel sample before and after soaking, respectively.

4.2.5 Interfacial bonding strength

4.2.5.1 Samples fabrication

Hydrogel sheets were prepared, each mimicking one layer in the bioprinted constructs. Three types of samples were fabricated, namely the 2-layered Alg3/MC9, the 2-layered Alg3/MC9-TSC, and the bulk Alg3/MC9, respectively. The 2-layered samples consisted of two layers of Alg3/MC9 hydrogels each with a thickness of 1 mm. The bulk Alg3/MC9 as a control was 2 mm in thickness. Each hydrogel sheet was fabricated between two glass slides that were wrapped with a cling film separated by a 1 or 2mm spacer.

The samples treated with and without TSC were named 2-layered Alg3/MC9-TSC, and 2-layered Alg3/MC9, respectively. In particular, the 2-layered Alg3/MC9-TSC sample was prepared as follows. One hydrogel sheet was treated with various concentrations of TSC solution using disposable Kimwipes (Sigma-Aldrich) to distribute the TSC solution evenly on one surface. As soon as the wiper was removed, the treated hydrogel sheet was placed immediately onto another hydrogel sheet to

produce a 2 mm-thick sample. The sample was kept in contact for a period, that is, contact time, for molecular rearrangement at the interface.

All of the prepared samples were immersed in a 100 mL CaCl_2 bath at room temperature for 10 min for post-crosslinking. The concentration of CaCl_2 in the bath was varied from 0 to 40 mg/mL. After the samples were removed from the bath, each sample was cut into dimensions of 20 mm by 20 mm (by 2 mm thickness) before lap-shear testing.

4.2.5.2 Effect of various parameters on the hydrogel-hydrogel interface

A parametric study was carried out to determine the key factors that affect the adhesion at the interface of layered hydrogels. Four sets of studies were performed: (1) 1 mL of a TSC solution with various concentrations (5, 10, 15, 20, 25, and 30 mg/mL) with a 6 min contact time and post-immersion in a CaCl_2 bath (20 mg/mL) for 10 min; (2) the TSC solution (15 mg/mL) with various volumes (0.5, 1, 1.5, and 2 mL), with a contact time of 6 min and post-immersion in a CaCl_2 bath (20 mg/mL) for 10 min; (3) Various TSC solution contact times (0, 2, 4, 6, 8, and 10 min) using 1 mL of the TSC solution (15 mg/mL), and post-immersion in a CaCl_2 bath (20 mg/mL) for 10 min; (4) 1 mL of the TSC solution (15 mg/mL) with 6 min contact time before post-immersing in the CaCl_2 bath with various concentrations (10, 20, 30, and 40 mg/mL) for 10 min. All these experiments were carried out at room temperature.

4.2.5.3 Lap-shear test

Lap-shear tests were carried out using an Instron machine (Instron 5569, UK) at room temperature with a 10 N load cell to investigate the interfacial properties between the hydrogel sheets. The samples were attached to the ends of the aluminum grips using cyanoacrylate glue. Once adhered to the grips, another short aluminum plate was attached to the other end of the long grips. A shear force was then applied at the hydrogel-hydrogel interface. During testing, the samples ($n = 6$) were pulled to failure at a displacement rate of 0.025 mm/s. The ultimate shear stress (USS), that is, the maximum shear stress up to which the sample resists failure in shear, was obtained from the stress-time curve.

4.2.6 Cyclic compression test

The mechanical properties of bulk Alg3/MC9 hydrogels, and 2-layered Alg3/MC9-TSC hydrogels were tested with a uniaxial compression tester (Instron 5569, UK) at room temperature with 10 N load cell. All the samples were prepared into a cylindrical shape with a diameter of 20 mm. Here, the bulk Alg3/MC9 was prepared with a height of 10 mm. Meanwhile, the 2-layered Alg3/MC9-TSC hydrogels were 5 mm thick in each layer, which were bonded using TSC to a final thickness of 10 mm. The cylindrical samples were soaked in a CaCl_2 bath (40 mg/mL) for 10 min. Mechanical testing was performed after quickly drying the samples' surface. The samples were subjected to two preloading cycles to 5% strain to eliminate

artifacts. The subsequent cyclic tests were recorded over six cycles at 10 and 30% strains, respectively. All tests were performed at room temperature and at a constant speed of 0.025 mm/s.

4.2.7 3D bioprinting of Alg3/MC9 hydrogel constructs

4.2.7.1 Cell culture

Mouse fibroblast L929 was cultured and expanded prior to bioprinting. The cells were cultured in the cell culture media of high glucose DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic, incubated under 5% CO₂ at 37 °C [134]. The cells were detached and counted before being loaded into a syringe of the bioprinter.

4.2.7.2 Bioprinting

In this study, the Alg3/MC9 hydrogel structures were bioprinted using the RegenHU bioprinter (see Figure 4.1), which is driven by pressure.

Figure 4.1 Image of RegenHU 3D bioprinter.

Here, two syringes were prepared for demonstration of L929 bioprinting. Syringe

1 was filled with the Alg3/MC9 hydrogel, while syringe 2 was loaded with L929 cells in a TSC solution. The Alg3/MC9 hydrogel was poured into syringe 1 at ~50 °C after mixing the MC powder into the hot Alg hydrogel. Syringe 1 was sealed and left sitting in a refrigerator at ~4 °C overnight. The cell suspension was added to the TSC solution, resulting in a final cell concentration of $\sim 3 \times 10^6$ cells/mL in the 15 mg/mL TSC solution.

The 3D bioprinting process was conducted at room temperature. The bioprinter was UV sterilized for ~1 hour before printing. The 3D printing route was generated from the 3D software (BioCAD) on the bioprinter to control the continuous 3D deposition of computer-designed patterns of hydrogels. In this study, multi-layered grids in a 0/90 ° pattern were printed out.

The inner diameter of the nozzles used for syringe 1 (with Alg3/MC9) and syringe 2 (with cells-TSC solution) were 0.25 mm (25 GA) and 0.21 mm (27 GA), respectively. The extrusion pressure used for printing with syringe 1 was 4 bar. For syringe 2, the least possible printing pressure (< 0.1 bar) was utilized for printing of the relatively low viscosity liquid (cells-TSC solution). The bioprinting of a 3D construct was performed layer-by-layer by extruding the hydrogel from syringe 1 followed by extruding the L929-TSC solution from syringe 2 onto a glass slide or a petri dish. All the printed constructs were post-submerged in a 40 mg/mL CaCl₂ bath for 10 min for crosslinking of the hydrogel. The CaCl₂ solution was then replaced with a warm cell culture media. The bioprinted constructs were cultured in the incubator

of 37 °C for up to 5 days. The detailed procedure for bioprinting of an Alg3/MC9 hydrogel construct is illustrated in Figure 4.2. In addition, a food dye was incorporated to better display the acellular bioprinted construct.

Figure 4.2 Schematic illustration of the extrusion-based bioprinting process with the Alg/MC hydrogel and cells-TSC solution. The construct is built layer by layer, wherein each layer is formed by extruding the Alg/MC hydrogel from syringe 1 followed by extruding a cells-TSC solution from syringe 2. The construct is post cross-linked in a CaCl_2 solution before culturing at 37 °C in a cell culture media.

4.2.8 Cell viability of the bioprinted Alg3/MC9 hydrogel construct

Immediately after bioprinting, cell viability of the bioprinted constructs was

examined using a live/dead assay (Molecular Probes) according to the reference [155]. Briefly, the bioprinted constructs were incubated in a DPBS solution containing 5 $\mu\text{mol/L}$ propidium iodide and 2 $\mu\text{mol/L}$ calcein acetoxymethyl ester for 15 min at 37 °C before examining via an inverted fluorescent microscope (Zeiss Axio Vert. A1). The cell viability, that was, the ratio of the number of live cells to the number of total cells, was computed manually from the fluorescence readings. For cell viability at day 3 and day 5, the bioprinted constructs were cultured in a humidified incubator before accessing the live/dead percentage. During culturing, cell culture medium was changed every 2 days. L929 cells were also cultured on tissue culture polystyrene (TCPS) as control.

4.2.9 Statistical analysis

All data were expressed as mean $\pm$ standard deviation (S.D.), and compared statistically by means of one-way ANOVA coupled with Tukey's test. Differences were statistically significant when $p \leq 0.05$.

4.3 Results

4.3.1 Rheological evaluation

4.3.1.1 Determination of shear thinning

In the steady-state flow test, it was found that the viscosity of all the tested hydrogels decreased with increasing shear rate, indicating a shear-thinning behavior.

It was also observed that across the entire range of shear rates applied, all of the Alg/MC hydrogels exhibited comparatively higher viscosity than Alg3 hydrogels (Figure 4.3 (a)). Interestingly, the viscosities over the range of the applied shear rates for MC1 and Alg3/MC1 were almost overlapping. MC3 and Alg3/MC3 as well as MC9 and Alg3/MC9 exhibited a similar behavior. These results indicate that the viscosities of the blend hydrogels are mainly contributed by MC. The flow behavior by gravity, as tested by an inverted test tube of hydrogel samples, is shown in Figure 4.3 (b), where an induced flow is observed for Alg3(i), MC1 (ii) and Alg3/MC1 (iii).

Figure 4.3 Rheological behaviors of Alg3 (i), MC1 (ii), Alg3/MC1 (iii), MC3 (iv), Alg3/MC3 (v), MC9 (vi), and Alg3/MC9 (vii). (a) Shear viscosity as a function of shear rate at room temperature. (b) Photographs showing the flow behavior of each hydrogel upon post transposing the hydrogel-containing tubes at room temperature for 5 min.

4.3.1.2 Determination of Shear rate

The optimum pressure for extruding each hydrogel using the bioprinter is listed in Table 1. Figure 4.4 shows the optical microscopic (OM) images of the printed filaments using different hydrogels. Diameters of extruded filaments decreased with increasing concentration of MC as shown in Figure 4.4. The printed MC1 and Alg3/MC1 showed comparable filament diameters. A similar trend was observed for MC3 and Alg3/MC3 as well as MC9 and Alg3/MC9.

Figure 4.4 OM images of printed filaments using different hydrogels. The filament thicknesses are indicated, where all the values shown are in μm . Alg3 (i), MC1 (ii), Alg3/MC1 (iii), MC3 (iv), Alg3/MC3 (v), MC9 (vi), and Alg3/MC9 (vii).

Assuming a uniform flow rate (V) of a non-Newtonian fluid flowing through an extrusion nozzle of inner radius R during the printing process, the volumetric flow rate (Q) of the fluid can be calculated as $Q = \pi R^2 V$ [123]. Table 4.1 shows the flow rate of hydrogel in the nozzle for each sample. In general, hydrogels with higher MC concentrations show a slower flow rate, which is another indication of their higher viscosities. When comparing Alg3/MC9 to Alg3, the latter was extruded out with a faster flow rate through the nozzle because of its low viscosity.

Table 4.1 Optimum printing pressures for hydrogels and computed flow rate of the hydrogels from a 0.25 mm nozzle

Parameters	Samples						
	Alg3	MC1	Alg3/MC1	MC3	Alg3/MC3	MC9	Alg3/MC9
Pressure (Bar)	0.3	0.8	0.8	2	2	4	4
Flow rate (mm/s)	156.7	12.0	11.1	10.2	9.0	8.6	7.6

The power-law index (n) was obtained from curve fitting based on the steady-state flow tests by using the power-law model as described in Chapter 3, where m is the power-law consistence. All the tested hydrogels showed that the n values were smaller than 1 (Table 4.2), again indicating that they have the shear-thinning properties. The maximum shear rate of each hydrogel in the nozzle were then calculated based on equation (3.9). The results are given in Table 4.2. The maximum shear rate in the nozzle for Alg3 was the highest among the tested hydrogels. The remaining hydrogels suffered from maximum shear rate of $\sim 500 \text{ s}^{-1}$ in the nozzle.

Table 4.2 The power-law index (n), and the maximum shear rate suffered by the hydrogels in a 0.25 mm nozzle.

Parameters	Samples						
	Alg3	MC1	Alg3/MC1	MC3	Alg3/MC3	MC9	Alg3/MC9
n	0.38	0.36	0.37	0.29	0.28	0.21	0.23
Shear rate (1/s)	7059.75	554.66	506.4	526.17	473.14	534.01	446.74

4.3.1.3 Characterization for thixotropic property

The thixotropic properties of MC3, Alg3/MC3, MC9 and Alg3/MC9 hydrogels were investigated. The viscosities of the Alg3, MC1 and Alg3/MC1 hydrogels are too low and hence the printed filaments show a poor shape fidelity (Figure 4.4). The images for the prepared samples of Alg3(i), MC1(ii) and Alg3/MC1 (iii) also indicate that these three samples flow easily (Figure 4.3 (b)) and are unable to maintain the shape of printed constructs. Hence, these hydrogels were not further studied as they are not appropriate for 3D bioprinting. Nevertheless, Alg3 was tested as a control. In step II, the samples were sheared under a shear rate of 500 s^{-1} simulating the extrusion of the hydrogels. Figure 4.5 shows the viscosity recovery behavior of the samples. The overlapping thixotropic behaviors between MC3 and Alg3/MC3 and between MC9 and Alg3/MC9 were observed.

Alg3/MC9 was of interest to us because of its high shape fidelity as shown in Figure 4.4. The initial viscosity of Alg3/MC9 was $\sim 8000 \text{ Pa}\cdot\text{s}$, which decreased sharply to $42 \text{ Pa}\cdot\text{s}$ upon application of a shear rate of 500 s^{-1} . After removing the high shear rate, the viscosity built up to $4400 \text{ Pa}\cdot\text{s}$ in about 30 s, which was $\sim 56 \%$ recovery of its initial viscosity. Moreover, after a longer time (60 s), the viscosity recovered to $4820 \text{ Pa}\cdot\text{s}$ ($\sim 60.5 \%$ of the initial value). Considering the thixotropic properties of the hydrogels together with the shape fidelity and stacking ability of the printed structures, Alg3/MC9 was chosen as the best candidate for bioprinting.

Figure 4.5 Shear thinning and recovery behavior of hydrogels at room temperature. The inset illustrates the printing process simulated by the rheological study: step I, before printing; step II, during printing; and step III, after printing.

4.3.2 Morphology of Alg3/MC9 hydrogel

The microstructure of Alg3, MC9 and Alg3/MC9 hydrogels are shown in Figure 4.6. From the top views, it was seen that Alg3 contained a uniform porous structure and all the pores were very similar in size and shape. MC9 had a smooth surface with smaller pore sizes than the Alg3. The Alg3/MC9 hydrogel also showed a smooth surface to that of MC9. Its pore sizes look like a combination of the pore sizes of big Alg3 and small MC9. The cross-sectional views of all of the samples reveal a porous structure. The microstructure of the Alg3/MC9 hydrogel was perceived to be a mixture of the microstructure of both Alg3 and MC9 hydrogels.

Figure 4.6 SEM images for top views and cross-sectional views of Alg3, MC9, and Alg3/MC9 hydrogels.

4.3.3 Interfacial bonding strength

The procedure of the lap-shear test is illustrated in Figure 4.7 (a). The bulk Alg3/MC9, the 2-layered Alg3/MC9-TSC, and the 2-layered Alg3/MC9 were tested and compared as shown in Figure 4.7 (b).

4.3.3.1 Comparison of sheared surfaces

The interfacial failure is an indication of the adhesion at the interface between

two layers of a hydrogel [20]. A bulk hydrogel often shows a rough, irregular failure surface reflecting the difficulty in separating the hydrogel into two parts. For the 2-layered Alg3/MC9-TSC hydrogel (Figure 4.7 (b)), an uneven fracture surface was seen throughout the sample, which was a similar failure pattern to the bulk gel. In contrast, the 2-layered Alg3/MC9 hydrogels (Figure 4.7 (b)), failed by delamination at the interface with a smoothly sheared surface. This corresponds to a weak adhesive property at the interface.

Figure 4.7 (a) Schematic illustration of the lap shear test procedure. The inset shows an image of the tested sample. (b) The images illustrating the failure surfaces of the tested samples.

4.3.3.2 Parameters affecting adhesive property of layered hydrogels

Figure 4.8 (a) illustrates the shear stress vs time curves of the hydrogel samples.

The ultimate shear stress (USS) can be obtained from the shear stress-time curve.

Figure 4.8 (a) Stress-time curves of tested samples. The 2-layered Alg3/MC9-TSC sample was treated with 1 ml of a TSC solution (15 mg/mL) and a contact time of 6 min, and finally submerged in a 20 mg/mL CaCl_2 bath. (b) Effect of volume and concentration of TSC on the layered interface of Alg3/MC9 hydrogels. * indicates a significant difference in USS ($p \leq 0.05$) when applying different TSC concentrations at the hydrogel interface compared to that of the control (2-layered Alg3/MC9). # indicates a significant difference in USS ($p \leq 0.05$) when applying different volumes of TSC compared to that of the control (2-layered Alg3/MC9). (c) Effect of contact time of the TSC solution (15 mg/mL) on the layered interface. (d) Effect of concentration of CaCl_2 in the post-crosslinking bath on the USS of the 2-layered Alg3/MC9, the 2-layered Alg3/MC9-TSC, and the bulk Alg3/MC9.

Figure 4.8 (b) demonstrates the effect of concentration and volume of the TSC solution on the adhesion property of Alg3/MC9-TSC hydrogels. It is found that USS increased with increasing TSC concentration and TSC volume up to 15 mg/mL and 1 mL, respectively. A peak stress value of ~8.49 kPa was reached at these parametric values. The further increase in TSC concentration and volume resulted in the decrease

of USS. When an excessive amount (e.g. 2 mL) of the TSC solution (15 mg/mL) was applied, the USS was significantly lower than that of the control, the 2-layered Alg3/MC9. Figure 4.8 (c) illustrates the effect of TSC solution contact time on the USS of 2-layered Alg3/MC9-TSC hydrogels. The TSC solution's contact time had little effect on the USS. USS slightly decreased when the TSC solution (15 mg/mL) was applied for more than 6 min. Generally, the interfacial strengths of all of the tested samples were enhanced with increasing CaCl_2 concentration in the final immersion bath, as illustrated in Figure 4.8 (d). It is observed that bulk gels had the highest interfacial strength among the samples. The 2-layered Alg3/MC9-TSC had a high USS close to the USS of bulk Alg3/MC9.

4.3.4 Structural integrity of Alg3/MC9 sample

The weight loss of the tested Alg3/MC9 hydrogel samples was recorded up to day 5, as shown in Figure 4.9. After day 5, the edges of the samples cracked and broken into small pieces such that they were not able to be lifted for weighing. The representative pictures of the samples during structural degradation are shown in Figure 4.9. The samples completely shattered after 21 days of incubation at 37 °C.

Figure 4.9 Structural integrity of Alg3/MC9 hydrogel in DI water at 37 °C.

4.3.5 Cyclic compression test

Figure 4.10 shows the cyclic compression curves of bulk Alg3/MC9 and 2-layered Alg3/MC9-TSC under maximum strains of 10 and 30%, respectively. The 2-layered Alg3/MC9-TSC exhibited a similar cyclic recovery performance with the bulk Alg3/MC9 hydrogel. Both the hydrogels were elastic and show an excellent recovery capability. After removing the compression force from the samples, the strains returned to 0% with a minimal hysteresis, indicating that the hydrogels could recover to its initial shape. There is no significant difference in cyclic recovery between the bulk Alg3/MC9 and the 2-layered Alg3/MC9-TSC after being compressed for six cycles. Although the hydrogels showed the excellent recoverability after each compression cycle, they also showed the dependence on both deformation history and strain. That was, (1) during the first set of six compression circles at 10% strain, the hysteresis cycle shifted down with the increase in the number of cycles; and (2) after

six cycles of compression at 10% strain, the hydrogels became weaker during the subsequent compressive cycles at 30% strain. The average compressive moduli of bulk Alg3/MC9 and 2-layered Alg3/MC9-TSC hydrogels were computed to be 11.11 kPa and 7.17 kPa, respectively.

Figure 4.10 Cyclic compressive stress-strain curves for 2-layered Alg3/MC9-TSC and bulk Alg3/MC9 hydrogels under maximum strains of 10 and 30%. Inset highlights complete recovery from a strain of 10%.

4.3.6 Printability of Alg3/MC9-TSC

Figure 4.11 (a) illustrates that the filaments printed using the Alg3/MC9 hydrogel possess the excellent regularity with a smooth surface. The width of the filaments was about 0.25 mm that conformed to the nozzle diameter of 0.25 mm. On the other hand, the filament printed using pure alginate (Alg3) had a much bigger width of around 0.50 mm. This implies that the Alg3/MC9 hydrogel exhibits a much higher shape

fidelity than the pure alginate hydrogel (Alg3). Figure 4.11 (b) shows the 3D printed 10-layered structures using Alg3 and Alg3/MC9 hydrogels. The Alg3/MC9 hydrogel exhibited an excellent printability, and the computer designed structure and shape were nicely maintained. In contrast, the printed Alg3 structure collapsed and could not form a 3D structure. The designed pores were also unable to be printed.

Figure 4.11 (a) OM images of the designed pore structure of the first layer of hydrogel constructs. The images are combined from multiple images of each sample captured under OM. (b) Pictures of the 3D printed hydrogel structures.

Printing of a hydrogel into a 3D construct is very challenging. Insufficient strength of the previously laid hydrogel will result in structural collapse. Vertical height of a printed construct could directly reflect the stackability of the hydrogel. Figure 4.12 (a) shows the printed Alg3/MC9-TSC constructs with different designs. The thickness of each printing layer was about 0.25 mm. The shapes of the constructs were stable, and the delicate internal porous structures were successfully fabricated. The spiral construct with 150 layers of printing was about 33 mm tall. In addition, the printed Alg3/MC9-TSC slab exhibited a high flexibility under bending and knotting

forces as shown in Figure 4.12 (b). Besides, the printed grid construct presented a great elasticity and recovery property. These results were in good agreement with the cyclic compression test.

Figure 4.12 (a) Pictures of a grid construct with 50 layers (height ~12 mm), a star construct with 100 layers (height ~24 mm), and a spiral construct with 150 layers (height ~33 mm). (b) Images of hydrogel slabs exerted with external forces.

4.3.7 Cell viability of Alg3/MC9-TSC

The cell viability of the bioprinted Alg3/MC9-TSC was examined immediately after bioprinting (D0) and at 3 days (D3) and 5 days (D5) of cell culturing, and the results are presented in Figure 4.13 (a).

All the tested samples showed a cell viability of more than 95%, which is comparable to that of the TCPS control. The L929 cell morphologies on TCPS and in bioprinted Alg3/MC9-TSC are shown in Figure 4.13 (b). At D0, L929 cells were rounded in Alg3/MC9-TSC. After culturing for 5 days, some of the cells became elongated showing a fibroblasts morphology. Meanwhile, cells were overcrowded on the TCPS control because the cell number cultured on the 2D TCPS surface was the same as in the 3D bioprinted hydrogel constructs. Cells had more space to proliferate in the bioprinted Alg3/MC9-TSC.

4.4 Discussion

Alg hydrogels are mechanically weak and could not maintain their 3D printed shapes. In this chapter, an appealing hydrogel was successfully obtained by simply blending Alg with MC. Compared to pure Alg3, this Alg/MC blend showed a higher viscosity, which is better for 3D printing. Previously, the gel network structure and thermoreversible gelation of MC in water were systemically studied by our group [156, 157]. The chemical structure of MC is characterized by the presence of both hydrophilic hydroxy (-OH) and hydrophobic methoxy groups (-OCH₃) [158, 159]. After adding MC to an Alg hydrogel, a semi-interpenetrating network-like structure is formed. The significantly high viscosity of Alg/MC blend hydrogels could be attributed to ionic crosslinking between Alg chains, hydrophobic interaction between MC molecules, hydrogen-bonding between -OH and -COOH groups, and

interpenetrating between Alg and MC networks [160]. Especially, the Alg3/MC9 hydrogel possessed the excellent thixotropic property and is an ideal 3D printable hydrogel. This thixotropic property is mainly contributed by the MC. As shown in Figure 4.5, although only half of the initial viscosity (4400 Pa·s) for Alg3/MC9 was recovered 30 s after removal of the shear rate (500 s⁻¹), the recovered viscosity value was still much higher than that (582 Pa·s) of a 10% Alg hydrogel, as discussed in Chapter 3. Furthermore, the reason for the viscosity of a hydrogel to recover after a period of rest is because the broken cross-links caused by shearing need some time to be rebuilt.

The Alg3/MC9 hydrogel showed a highly porous and interconnected microstructure. Mooney et al.[75] studied the properties and structure of Alg-based hydrogels. They reported that Alg hydrogels are ideal for the migration of living cells because of its interconnected porous structure. Our previous study [71] reported that the size and density of pores in Alg hydrogels could be controlled by changing the concentration of Ca²⁺ ions. Introduction of MC into Alg could modify its microstructure and control its pore size by interpenetration of two polymer networks and adjusting the Ca²⁺ ions content.

There are layer defects in 3D bio-printed constructs due to the layer-by-layer printing. Our strategy proposed to improve the interfacial bonding between printed layers of Alg3/MC9 hydrogels was to use a TSC solution, as explained in Figure 4.14. TSC was chosen instead of other chelating agents such as ethylenediaminetetraacetic

acid (EDTA) and citric acid because it is relatively biocompatible [161]. Making use of the physical crosslinks between Ca^{2+} ions and Alg chains, the TSC solution was applied at the layered interface to remove the Ca^{2+} ions from the applied surfaces. The subsequent post-cross-linking of the hydrogel constructs in the CaCl_2 bath created the interfacial connection between layers and improved the interfacial bonding strength. In fact, the 2-layered Alg3/MC9-TSC hydrogel demonstrated a higher USS than the 2-layered Alg3/MC9 in the lap-shear test. This can be attributed to the fact that there are interlaminar cross-links. For the 2-layered Alg3/MC9, the CaCl_2 post-cross-linking bath primarily helped in forming of intralaminar cross-links within each layer. The cyclic compressive test results showed that both the 2-layered Alg3/MC9-TSC hydrogel and the bulk Alg3/MC9 hydrogel exhibited the excellent recovery property. But the mechanical performance of the TSC-treated hydrogel is not significantly different to the bulk Alg3/MC9 hydrogel. Moreover, the low viscosity TSC solution can be used to deposit cells in each layer, whereas it was difficult to load cells into the highly viscous Alg3/MC9 hydrogel. Therefore, in this study, TSC has been verified to possess two functions: an interfacial bonding improving agent and a bioink medium for loading cells for 3D bioprinting.

Figure 4.14 Schematic illustrating the strengthening mechanism at the Alg/MC hydrogel interface using a TSC solution.

The key parameters affecting the interfacial bonding strength of a 3D printed Alg3/MC9 construct are the concentration of TSC, the volume of TSC and the concentration of CaCl_2 in the post-cross-linking bath. Appropriate concentration and volume of TSC could enhance the adhesion between layers of Alg3/MC9. However, a higher concentration or excessive volume of TSC might lead to an opposite effect because excessive Ca^{2+} ions throughout the hydrogel might be removed. It has also been found that contact or retention time of the TSC solution on the interlaminar surface of the printed Alg3/MC9 construct did not have a significant effect on the enhancement of interfacial bonding. Once the Ca^{2+} ions are chelated at the interface, further retention of the TSC solution does not cause further removal of Ca^{2+} ions. On the contrary, a short contact time of TSC is desirable for 3D bioprinting of the living cells and an Alg/MC hydrogel to make constructs continuously layer-by-layer.

In addition to the rheological evaluation, the printability of a hydrogels can also be evaluated from the shape fidelity of a printed construct. A high regularity and a high resolution in printed filaments, edges, and corners are all indications of a good printability. Furthermore, it is important to have the printed 3D shapes to be consistent with the designed structures, where stackability of the hydrogels comes into play. The printed Alg3 hydrogel showed an inferior shape fidelity even in the first layer, and the subsequent printing could not be proceeded well. Meanwhile, printing of Alg3/MC9 resulted in the consistent filaments with a high 3D shape fidelity. The 3D constructs with an over 33 mm height could be printed, wherein the high shape fidelity was

sustained until the 150th layer. The pressure used for printing Alg3/MC9 hydrogel was about 4 bar, which is almost the maximum possible printing pressure of the used bioprinter. However, the time for obtaining a 50 layered grids structure was more than 4 hours. Thus, a higher pressure is favorable for quickly fabrication multilayers structures through printing highly viscous hydrogel.

3D bioprinted Alg3/MC9-TSC construct had an excellent biocompatibility with a cell viability of more than 95% up to day 5. The cells started to elongate inside and on the surface of the hydrogel after culturing for 5 days, indicating that the normal fibroblastic morphology was retained. The Alg3/MC9 hydrogel was hydrolytically degradable, which allowed the printed cells to build their own extracellular matrices (ECM). Schütz et. al. [78] reported that MC is completely released from Alg/MC mixture hydrogels within 7 days. Because Alg3 is weak, the strength of the cultured construct should be eventually maintained by the cellular ECM. In this chapter, a strategy was reported to bioprint construct with improved adhesion at the interface. Printing of complex and tall constructs with excellent shape fidelity and sufficient mechanical stability was achieved. The Alg/MC blend hydrogel is easily obtained, inexpensive, and presents excellent biocompatibility, which can broaden the application of such materials and methods to the field of 3D bioprinting and tissue engineering.

4.5 Summary

In this chapter, a novel and 3D printable hydrogel blend (Alg/MC) was reported. In addition, an interfacial bonding agent (TSC) was successfully introduced to significantly improve the interfacial adhesion between printed layers of the hydrogel. The rheological properties of the Alg/MC hydrogels before, during and after printing were investigated as a function of hydrogel composition. The best hydrogel composition for the best 3D printability was found to be the Alg3/MC9 hydrogel consisting of 3 wt% alginate and 9 wt% MC. The interfacial bonding strength of the layered Alg3/MC9 hydrogel was significantly improved by a TSC solution. The TSC solution acted as a chelating agent to remove the interfacial calcium ions. The subsequent cross-linking in a CaCl_2 bath built the cross-links between Ca^{2+} ions and Alg chains, which promoted interfacial bonding between layers of hydrogels. The concentration of TSC, the volume of TSC and the concentration of CaCl_2 in the post-crosslinking bath were the major factors to enhance the interfacial bonding strength of 3D printed constructs of the Alg3/MC9 hydrogel. As an exciting result, the Alg3/MC9 hydrogel, with the help of TSC, could be printed into different 3D constructs with up to 150 layers (or about 33 mm high), and it also showed the excellent flexibility in terms of elasticity and bending strength. Finally, the TSC solution with low viscosity was utilized to load and co-print cells into a 3D construct made by the Alg3/MC9 hydrogel with the aid of TSC. The bioprinted Alg3/MC9

hydrogel together with L929 cells-TSC exhibited a high biocompatibility. L929 cells retained their fibroblast morphology in the hydrogel, and the cell viability was more than 95% at day 0, day 3 and day 5 of culturing. Additionally, the Alg3/MC9 hydrogel was hydrolytically degradable at 37 °C. In conclusion, the Alg3/MC9-TSC is an ideal bioink with high printability and good biocompatibility for bioprinting. The obtained Alg/MC-TSC construct had the strong interface bonding.

Chapter 5 3D Printing of Oppositely Charged Hydrogels with Super Strong Interface Bonding

This chapter focuses on proposed a strategy for utilizing two oppositely charged hydrogels for 3D printing constructs with strong interfacial bonding.

5.1 Experimental design

In terms of ionic charge, hydrogels can be neutral (e.g. dextran), anionic (e.g. alginate (Alg), xanthan(Xan), κ -carrageenan (Kca)) and cationic (e.g. chitosan (Chi), gelatin (Gel), GelMA) [162, 163]. Natural hydrogels, such as Alg [12, 79], Gel [138, 139], collagen [15, 164], and chitosan [18], which show good biocompatibility with nontoxic degradation products, have received great attention in the field of biomedical engineering. However, these natural hydrogels still have limitations for their broad applications as discussed in Chapter 2. Various strategies have been reported for developing hydrogels with good mechanical strengths. For example, the double network hydrogels can sustain large deformation and force without failure[165, 166]. However, the double network hydrogels often contain a chemical network that is made by UV curing or a chemical process which poses a potential risk for utilizing these hydrogels in biomedical fields, as their degradation product is likely to be toxic [9, 111].

As 3D printing is a layer-by-layer printing process, there are often layer defects

or weak interface adhesion in a 3D-printed layered structure [20, 21]. As a new approach, alternate printing of two kinds of hydrogels maybe overcome the drawbacks of printing one hydrogel alone. Furthermore, alternate printing of two oppositely charged ionic hydrogels is expected to result in a strong interface adhesion between layers. However, such method has not been reported in the literature.

In this chapter, a promising approach was reported that is capable of printing a 3D construct with strong interfacial bonding by utilizing the ionic interaction between two oppositely charged hydrogels. Alg, Xan, and Kca are chosen as the representatives of anionic hydrogels, and Chi, Gel, and GelMA are chosen as those of cationic hydrogels, to find the optimal combination of two oppositely charged hydrogels for the best 3D printability with strong interface bonding. Specific properties, including rheological properties of the prepared hydrogels, shape fidelity of a printed structure, and structural integrity of a printed construct in cell culture medium, are studied as functions of polymer concentration and the combination of hydrogels.

5.2 Materials and methods

5.2.1 Materials and sample preparation

Sodium Alg (with guluronic acid block content of 50-60%, Sigma-Aldrich, Singapore) was dissolved in a DPBS solution. The mixture was magnetically stirred overnight at room temperature to obtain a homogeneous hydrogel. The prepared Alg hydrogels were named Alg14, Alg16, Alg18, and Alg20, corresponding to the Alg

concentrations of 14%, 16%, 18%, and 20% (wt/wt) in the DPBS solutions respectively.

Xan gum (from aerobic fermentation, Sigma-Aldrich, Singapore) hydrogels were prepared by gradually adding Xan powder into a DPBS solution while simultaneously stirring the mixture. The hydrogels Xan4 (4 wt% Xan), Xan5 (5 wt% Xan), Xan6 (6 wt% Xan), and Xan7 (7 wt% Xan) were prepared.

Kca (MW 3.0×10^5 g/mol, Sigma-Aldrich, Singapore) powder was gradually added to a hot DPBS ($\sim 80^\circ\text{C}$) solution. Meanwhile, the mixture was thoroughly stirred to obtain a homogeneous hydrogel. The hydrogels Kca1 (1 wt% Kca), Kca1.5 (1.5 wt% Kca), Kca2 (2 wt% Kca), and Kca2.5 (2.5 wt% Kca) were prepared.

Chi (medium molecular weight, Sigma-Aldrich, Singapore) powder was gradually added in the DPBS, where the pH of the solution was previously adjusted to 3 by adding acetic acid dropwise. Next, the mixture was stirred at room temperature until the Chi powder was evenly dissolved. The hydrogels Chi4 (4 wt% Chi), Chi5 (5 wt% Chi), Chi6 (6 wt% Chi), and Chi7 (7 wt% Chi) were prepared.

Gel powder (type A from porcine skin, Sigma-Aldrich, Singapore) was gradually adding into DPBS at about 50°C . The mixture was thoroughly stirred until the gelatin powder was evenly dissolved. The hydrogels Gel6 (6 wt% Gel), Gel7 (7 wt% Gel), Gel8 (8 wt% Gel), and Gel9 (9 wt% Gel) were prepared.

The synthesis of GelMA was carried out according to the literatures [167, 168]. Gel (10 g) was dissolved into a 100 mL DPBS solution and stirred until fully dissolved.

0.7 mL of methacrylic anhydride (MA) (Sigma-Aldrich, Singapore) was added into the Gel solution while stirring. The reaction proceeded at 50 °C for 3~4 hours. The mixture was dialyzed at 37 °C against DI water using a 12-14 kDa membrane tubing for one week. Then the solution was moved to 50 mL tubes and freeze dried for another one week. The dry GelMA was stored at -40 °C until further use. GelMA with different amounts was dissolved in DPBS to obtain GelMA hydrogels with different concentrations. GelMA8 (8 wt% GelMA), GelMA9 (9wt% GelMA), GelMA10 (10 wt% GelMA), and GelMA11 (11wt% GelMA) were prepared. The photo initiator (PI) 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Sigma-Aldrich, Singapore) was added in the hydrogel with a PI/GelMA solution ratio of 0.02 g: 9 mL.

5.2.2 ¹H nuclear magnetic resonance characterization

The degree of methacrylation (DM) of GelMA was measured using ¹H nuclear magnetic resonance (NMR) spectroscopy (AV300 NMR). Three spectra were repetitively collected from each sample. Phase correction was applied before obtaining the purely absorptive signals. The areas of the peaks of interest were integrated after baseline correction. The *DM* was defined as follows [169],

$$DM(\%) = \left(1 - \frac{Area(lysine\ methylene\ of\ GelMA)}{Area(lysine\ methylene\ of\ Gelatin)} \right) \times 100\% \quad (5.1)$$

5.2.3 Rheological measurement

The rheological properties of the hydrogels with various concentrations were

measured using a rotational rheometer (DHR, TA Instruments, USA) with a 40 mm parallel plate and a 0.55 mm measurement gap. Two rheological tests at 26 °C (the working temperature of the 3D printer) were adopted: (1) steady-state flow tests in a range of shear rate 0.5-500 s⁻¹; (2) recovery tests under a calculated shear rate simulating the extrusion process for 3D printing as described in 3.3.2.1.

5.2.3.1 Determination of shear rate

In an extrusion nozzle, the shear rate exerted on a hydrogel at a radial position [123], r ($0 < r < R$) could be estimated by a deduced equation (3.9): The inner diameter of the nozzle used in this chapter was 0.25 mm. The details for obtaining the flow rate of each sample were provided in Chapter 4 (section 4.2.2.2).

5.2.3.2 Characterization of thixotropic property

The rheological properties of each hydrogel before (step I), during (step II), and after (step III) the extrusion process was simulated. Step I simulated the initial state of a hydrogel before printing where a shear rate of 0.1 s⁻¹ was applied and held for 60 seconds. At step II, a shear rate, which was calculated based on equation (3.9), was applied on the hydrogels for 10 seconds. This step simulated the state of a sheared hydrogel during the extrusion process. At step III, the shear rate was decreased to 0.1 s⁻¹ again and held for another 60 seconds to simulate the final condition of the hydrogel after extrusion.

5.2.4 Evaluation of printability of each hydrogel

5.2.4.1 Determination of the best concentration of each hydrogel

The smooth surface and constant width of a printed filament, which resulted in regular edges and corners in the printed 3D construct, are all indications of a good printability. Moreover, the printed 3D shapes should be consistent with the pre-designed pattern. Thus, the printability of a hydrogel can be investigated from the shape fidelity of a printed construct.

5.2.4.2 Determination of the best hydrogels for printing

The construct printed with an anionic hydrogel then a cationic hydrogel alternately, was named as anionic-cationic, i.e. Alg18-Gel5. In this study, a bioprinter (BioFactory bioprinter machine, RegenHU) was used to print 20-layered grids. The printing pressure utilized for each hydrogel is listed in Table 5.1. The optimal pressure for extruding each hydrogel was obtained when a continuous filament could be deposited with a uniform filament diameter. The i.d. of the nozzle used was 0.25 mm. The structures printed using each cationic or anionic hydrogel solely were also demonstrated as a control. The working temperature for the bioprinter was ~26 °C.

5.2.5 Measurement of interfacial bonding strength

5.2.5.1 Evaluation of interaction between two opposite charged hydrogels

A simple experiment was performed to investigate the interaction between an

anionic hydrogel and a cationic hydrogel. GelMA10 hydrogel (stained with orange-colored food dye) and Kca2 hydrogel (stained with blue-colored food dye) were cut into small pieces, respectively. Subsequently, pieces of hydrogels were placed next to each other alternatively, i.e. Kca2-GelMA10. The same types of hydrogels pieces, i.e. GelMA10-GelMA10, and Kca2-Kca2 were also investigated as a control. The Gel8-Kca2 were also prepared to investigate the interaction between a Gel8 hydrogel and a Kca2 hydrogel.

5.2.5.2 Quantitative study of interfacial bonding strength

The interfacial bonding strength between two hydrogel layers was investigated through lap-shear tests using an Instron machine (Instron 5569; U.K.) with a 10 N load cell. 2 mm thick hydrogel sheets were cast, each mimicking one layer in a bioprinted construct. Three types of samples were fabricated, namely 2-layered Kca2, 2-layered GelMA10, and 2-layered Kca2-GelMA10. In particular, the 2-layered Kca2-GelMA10 was prepared by placing one Kca2 hydrogel sheet immediately onto a GelMA10 hydrogel sheet to produce a 4 mm thick sample. The ultimate shear stress (USS), was recorded from the stress-time curve. The 2-layered Gel8 and 2-layered Kca2-Gel8 were also prepared and investigated.

5.2.6 Structural integrity of the printed constructs in 37 °C DPBS

The structure integrity of the printed Kca2-Gel8, and Kca2-GelMA10 construct were observed for 30 days after soaking it in DPBS in an incubator (37 °C). The

constructs solely printed using Kca2 or Gel8 or GelMA10 served as a control. Each structure had ten layers. The blend hydrogel (a mixture of Kca2 and GelMA10 in a volume ratio of 1:1) was cast and investigated in DPBS. The hydrogels were stained using a food dye for ease of visualization.

5.2.7 3D bioprinting of Kca2-GelMA10 hydrogel constructs

5.2.7.1 Bioprinting

Mouse myoblasts cells C2C12 were cultured in the cell culture media of high-glucose DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic, incubated under 5% CO₂ at 37 °C [134]. The cells were detached and counted before being loaded into a syringe for bioprinting. The cell suspension was added to the GelMA10 hydrogel, resulting in a final cell concentration of $\sim 3 \times 10^5$ cells/mL in the hydrogel.

Two syringes were utilized for bioprinting. Syringe 1 was loaded with the Kca2 hydrogel, while syringe 2 was filled with the cell-laden GelMA10 hydrogel. The 3D cell-laden construct was printed layer-by-layer by extruding the Kca2 from syringe 1, then followed by printing the cell-laden GelMA10 hydrogel from syringe 2. The freshly bioprinted Kca2-GelMA10 constructs were UV cured for 10 seconds in a UV flood (Shuttered UV system, Epoxy and equipment technology Pte Ltd). Then the constructs were cultured in an incubator at 37 °C for 5 days. Figure 5.1 illustrates the procedure for bioprinting of a Kca2-GelMA10 hydrogel construct.

Figure 5.1 Schematic illustration of the bioprinting procedure. The 3D cell-laden construct is printed layer-by-layer by printing the Kca hydrogel from syringe 1, then followed by printing the cell-laden GelMA hydrogel from syringe 2.

5.2.7.2 Cell viability of the bioprinted Kca2-GelMA10 hydrogel construct

The bioprinted constructs were cultured in the medium in an incubator (37 °C; 5% CO₂) for up to 5 days. The cell culture medium was changed every 2 days. The viability of cells in the constructs was examined using a live/dead assay (Molecular Probes) via an inverted fluorescent microscope (Zeiss Axio Vert. A1). The constructs were incubated in a DPBS solution containing 5 µmol/L propidium iodide and 2 µmol/L calcein acetoxymethyl ester for 15 minutes before fluorescence imaging. C2C12 cells were also cultured on TCPS as a control.

5.2.8 Statistical analysis

All results were presented as the mean $\pm$ standard deviation (S.D.), and compared statistically by means of one-way ANOVA. Differences were statistically significant

when $p \leq 0.05$.

5.3 Results and discussion

5.3.1 ^1H NMR characterization

Figure 5.2 shows the chemical structures of the unmodified gelatin, and GelMA. Compared to the unmodified gelatin (see Figure 5.2 (a)), the tested GelMA (see Figure 5.2 (b)) sample contains the new functional groups, which are marked as “a” and “c”. The ^1H NMR spectra verified the formation of these two functional groups, as shown in Figure 5.2 (c). Meanwhile, the peaks b indicates the signal of methylene in lysine groups of gelatin and GelMA. As lysine is the reactant, the intensity of peak b could be used to quantify DM. On the basis of equation (5.1), the DM of GelMA in this study was about 26%.

Figure 5.2 The chemical structures of (a) gelatin and (b) GelMA, and (c) their respective ^1H NMR spectra. Peaks a and c represent the signals of the grafted methacrylic group, and peak b indicates the signal of methylene in lysine groups of gelatin and GelMA.

5.3.2 Rheological evaluation

5.3.2.1 Determination of shear thinning and shear rate

As is well-known, a highly viscous hydrogel with good printability for an extrusion-based printer should be shear thinning [4]. Thus, all the tested hydrogel samples had one of the essential properties desired for the successful printing, as shown in Figure 5.3.

Figure 5.3 Shear viscosity as a function of shear rate. (a) Anionic hydrogels: Alg (i), Xan (ii), and Kca (iii). (b) Cationic hydrogels: Chi (i), Gel (ii), and GelMA (iii).

The procedure for obtaining the flow rate, and the power-law index (n) were described in Chapter 3 (see section 3.2.2). The results are listed in Table 5.1. It is well known that, for a shear-thinning fluid, the value of n should be smaller than 1 [123]. The value of n for each tested hydrogel was smaller than one (see Table 5.1), again revealing that they are shear-thinning hydrogels.

5.3.2.2 Characterization of thixotropic property

Based on equation (3.9), almost all the tested samples (except Kca 2.5) were estimated to be sheared under a maximum shear rate of about 100 s^{-1} during printing (see Table 5.1). The estimated value of shear rate exerted on a hydrogel was used for simulating the behaviors of a hydrogel during the extrusion process. The thixotropic properties of anionic hydrogels (Figure 5.4 (a)) and cationic hydrogels (Figure 5.4 (b)) were investigated. At step II, all the hydrogels were tested under a shear rate of 100 s^{-1} , which simulated the condition for the hydrogels to bear the shear force during the extrusion process. After that, moving to step III, each hydrogel recovered its viscosity to a comparable value of its initial viscosity (step I). All the tested hydrogels exhibited a thixotropic property. The reason for the changing of viscosity is because the cross-links or entanglements between polymer chains are broken by shearing, which resulted in a decrease in the viscosity of hydrogels (step II). After removing the high shear rate, the hydrogel could rebuild the broken cross-links after a period of rest (step III).

Table 5.1 The Optimum printing pressure for printing each hydrogel, flow rate, power-law index (n), and the maximum shear rate suffered by the hydrogels in a 0.25 mm nozzle

Anionic hydrogels												
Parameters	Alg14	Alg16	Alg18	Alg20	Xan4	Xan5	Xan6	Xan7	Kca1	Kca1.5	Kca2	Kca2.5
Pressure (Bar)	0.3	0.3	0.4	0.4	0.5	0.5	0.6	0.6	0.4	0.7	0.8	1.0
Flow rate (mm/s)	2.6	2.6	2.6	2.6	1.61	1.61	1.61	1.61	1.56	1.56	1.56	1.56
n	0.65	0.64	0.55	0.53	0.15	0.15	0.16	0.17	0.29	0.19	0.15	0.03
Shear rate (1/s)	94.4	94.9	100.21	101.65	124.51	124.51	119.14	114.41	80.47	103.12	120.64	453.44

Cationic hydrogels												
Parameters	Chi4	Chi5	Chi6	Chi7	Gel6	Gel7	Gel8	Gel9	GelMA8	GelMA9	GelMA10	GelMA11
Pressure (Bar)	0.2	0.3	0.4	0.4	0.6	0.7	0.8	0.9	0.7	0.7	0.8	1.0
Flow rate (mm/s)	2.06	2.06	2.06	2.06	1.62	1.62	1.62	1.62	1.05	1.05	1.05	1.05
n	0.62	0.51	0.45	0.34	0.19	0.18	0.17	0.14	0.06	0.07	0.08	0.07
Shear rate (1/s)	76.02	81.75	86.06	97.91	107.09	110.88	115.11	131.45	165.20	145.20	130.20	145.20

Figure 5.4 Rheological measurements to simulate the shear thinning and recovery behaviors of different hydrogels with various concentrations: step I, at a shear rate of 0.1 s^{-1} ; step II, at a shear rate of 100 s^{-1} ; step III, at a shear rate of 0.1 s^{-1} . (a) Anionic hydrogels: Alg (i), Xan (ii), and Kca (iii). (b) Cationic hydrogels: Chi (i), Gel (ii), and GelMA (iii).

5.3.3 Evaluation of the printability of hydrogels

5.3.3.1 Determination of the best concentration of each hydrogel

In this section, the shape fidelity of each hydrogel was investigated to find the best concentration for printing. For one-layer of grids in a 0°/90° pattern (used in this study), regular grids and square holes should be consistent with the designed pattern and dimensions if the hydrogel used has a good printability. Ouyang et al.,[130] reported that the extruded filaments would form a pattern with circular-like holes when the hydrogel is in an undergelation state. But when the hydrogel is in an overgelation state, the extruded filament is irregular or even shows fracture with a rough surface. Thus, each polymer with a different polymer concentration should be under one of the gelation states: undergelation, proper-gelation, or overgelation. The hydrogel in a proper-gelation state is suitable for printing. Thus, to find the best concentration of each polymer is essential before using this polymer for printing.

The printability (P_r) of a hydrogel can be defined according to the printed square shape [130]:

$$P_r = \frac{\pi}{4C} = \frac{P_L^2}{16A} \quad (5.2)$$

where P_L is the perimeter of one grid in the printed pattern and A is the area of the grid. P_L and A of one grid in the printed construct can be computed using the ImageJ software. C is the circularity of an enclosed area, is defined as $C = \frac{4\pi A}{P_L^2}$. It is known that a circle and a square have a circularity value of 1 and $\pi/4$, respectively. Thus, for

a hydrogel with an ideal printability, the interconnected channels of the printed constructs would demonstrate a square shape so that the value of P_r should be close to 1. $P_r < 1$ indicates that the hydrogel is in an undergelation condition. For $P_r > 1$, the hydrogel is in an overgelation condition. Based on the criteria, the best concentration of each polymer can be obtained.

Figure 5.5 illustrates the P_r of each hydrogel with various concentrations. For Alg hydrogels (Figure 5.5 (a-i)), irregular shape and obvious spreading were easily observed when the Alg hydrogels had comparatively low concentrations (Alg14 and Alg16). Although Alg20 has the highest concentration of Alg, it exhibited a similar P_r with Alg18. As the Alg powder was difficult to dissolve in DPBS when the concentration of Alg reached 20 %, 18wt % was chosen as the best concentration for Alg hydrogel for printing although its P_r is not ideal ($P_r < 1$). The Chi hydrogels demonstrated the similar issue of Alg. These results indicate that Alg and Chi are not ideal bioinks for 3D printing, because the P_r of these two types of hydrogels cannot reach 1 although they were already prepared with high concentrations. Finally, the hydrogels with the optimal concentrations were found to be the Alg18, Xan6, Kca2, Chi6, Gel8 and GelMA10 (Figure 5.5).

Figure 5.5 Evaluation of P_r of each hydrogel. A) Anionic hydrogels: Alg (i), Xan (ii), and Kca (iii). B) Cationic hydrogels: Chi (i), Gel (ii), and GelMA (iii). The inserts demonstrate the printed one-layer grids with different P_r . Note: The scale bar shown is 2 mm.

5.3.3.2 Determination of the best combination for printing

The 3D constructs printed using the selected hydrogels are illustrated in Figure 5.6 (a). The structures fabricated with Alg18 and Chi6 collapsed, all the printed filaments completely fused together, and the designed internal porous structure could

not be successfully maintained. The results indicate that these two hydrogels are not suitable for independently printing a 3D construct. In contrast, the structures printed using the rest hydrogels (Xan6, Kca2, Gel8 and GelMA10) all exhibited a good shape fidelity as compared to the structures fabricated using Alg18 or Chi6.

Instead of printing with a single hydrogel, the 3D printing results using three combinations (i.e. Chi, Gel, and GelMA groups) of two oppositely charged hydrogels are shown in Figure 5.6 (b). The printed structures of the Chi group (Alg18-Chi6, Xan6-Chi6 and Kca2-Chi6) cannot form a 3D shape. The printed structures with Alg18-Gel8 or Alg18-GelMA10 showed an inferior shape fidelity and the interconnected pores fused together even with the help of Gel8 or GelMA10. The results indicated that the combinations including Alg18 or Chi6 all exhibited a poor shape fidelity. This is because Alg18 or Chi6 was not suitable for solely printing a structure as discussed previously (see Figure 5.6 (a)), and the filaments printed using Alg18 or Chi6 were completely spread, which further affected the subsequently printed layer, and then the whole structure. In contrast, the shapes of the constructs fabricated using Xan6-Gel8, Kca2-Gel8, Xan6-GelMA10 and Kca2-GelMA10 were well consistent with the designed structures, wherein each filament was printed with a high regularity and a high resolution. But the filaments of Kca2-Gel8 and Kca2-GelMA10 were thinner and much regular than Xan6-Gel8 and Xan6-GelMA10, respectively. Thus, the optimal combinations for printing two opposite charged hydrogels were found to be the Kca2-Gel8 and Kca2-GelMA10.

Figure 5.6 (a) Pictures of 20-layered constructs printed with single hydrogels. (b) Images of the 20-layered constructs printed with an anionic hydrogel then a cationic hydrogel alternately. The scale bar shown is 5 mm.

5.3.4 Measurement of the interfacial bonding strength

5.3.4.1 Evaluation of interaction between Kca2 and GelMA10

From Figure 5.7 (a), it is observed that the GelMA10 and GelMA10 hydrogel pieces could not be lifted together after putting them together. The adhesion property of the hydrogel at the interface could not be further improved with a longer contacting time (2 hours). The results reveal that there is no sufficient adhesion between two GelMA10 pieces. A similar trend is observed between two Kca2 pieces. In contrast,

the Kca2 and GelMA10 hydrogel pieces could adhere to each other immediately and could be lifted together against their own weight, indicating that there is a strong attraction between GelMA10 and Kca2. Figure 5.7 (b) further demonstrates the adhesion properties between Kca and GelMA. Especially, the experiment shown in Figure 5.7 (b-iv) proved the extraordinary adhesion between GelMA10 and Kca2, whereby more than 18 hydrogel pieces could be attached alternately and lifted against their own weight (total weight of ~ 2.8 g). It is also observed that there is an attraction between Gel8 and Kca2 hydrogel pieces (Figure 5.7 (c)).

Figure 5.7 Photographs demonstrating interactions between hydrogels. (a) GelMA10 and GelMA10 or Kca2 and Kca2 cannot be lifted up against their own weights. Once put together, Kca2 and GelMA10 are attached alternately and lifted against their own weight. (b) Images demonstrating extraordinary adhesion between Kca2 and GelMA10. (c) Images illustrating interactions between Gel8 and Kca2.

Figure 5.8 illustrates the molecular structures of Kca and GelMA. The Kca molecules are negatively charged in aqueous solution, because they have one negatively charged sulphonic acid group per carrabiose unit [170]. GelMA was synthesized from Gel [167]. The inherent cationic character of Gel is due to the presence of arginine and lysine residues [162]. According to the results of ^1H NMR spectroscopy, the DM of GelMA in our study was about 26 % (section 5.3.1), which indicated that only 26% methylene in lysine groups participated in the reaction and the rest 74% made GelMA exhibited a cationic character. The adhesion between the Kca2 and GelMA10 could be attributed to the electrostatic interactions between Kca2 and GelMA10 and the hydrogen bonds between $-\text{OH}$ and $-\text{COOH}$ groups [171].

Figure 5.8 The molecular structures of GelMA and Kca. A schematic illustration for the interaction between GelMA and Kca hydrogels.

5.3.4.2 Quantitative study of interfacial bonding strength

Figure 5.9 (a) shows the procedure for the lap-shear test. The interfacial failure pattern is an indication of the adhesion property at the interface of a layered construct.

The 2-layered Kca2 and 2-layered GelMA10 hydrogels failed by delamination at the interface with a smooth surface, reflecting a weak adhesion at the interface. In contrast, an uneven and rough failure surface was seen throughout the 2-layered Kca2-GelMA10, indicating a good adhesion between layers.

Figure 5.9 (a) Schematic and photographic illustrations of the lap-shear test procedure. The images on the right side show the failure surface of samples. (b) Stress-time curves of the tested samples (i), and USS of the tested hydrogels (ii). * indicates a significant difference in USS ($p \leq 0.05$).

The shear stress versus time curves of the representative samples are illustrated in Figure 5.9 (b-i). The USS of 2-layered Kca2-GelMA10 hydrogels was significantly higher than the other two samples (Figure 5.9 (b-ii)). The results of the lap-shear test for 2-layered Kca2, 2-layered Gel8 and 2-layered Kca2-Gel8 are shown in Figure 5.10. The 2-layered Kca2-Gel8 presented a good adhesion between layers, as compared to 2-layered Kca2 and 2-layered Gel8.

Figure 5.10 Quantitative study of interfacial bonding strength between Kca2 and Gel8. USS of the tested samples. * indicates a significant difference in USS ($p \leq 0.05$).

5.3.5 Structural integrity of the printed constructs in 37 °C DPBS

The structural stability and integrity for the 3D printed Kca2, Gel8, and Kca2-Gel8 constructs in 37 °C DI water were investigated (see Figure 5.11). The 3D printed Kca2 and Gel8 constructs were completely dissolved after being incubated in DI water (37 °C) within 1 hour. In contrast, the 3D printed Kca2-Gel8 hydrogel construct exhibited a high structural integrity for up to 120 hours (5 days) in DI water. This result indicates that 3D printing of oppositely charged hydrogels can improve the

structural integrity of a 3D construct, compared to the constructs printed with an anionic hydrogel or a cationic hydrogel solely.

Figure 5.11 Structural integrity of Kca2-Gel8 constructs in 37 °C DI water.

After that, the structural stability for the printed constructs was examined in 37 °C DPBS, as shown in Figure 5.12. After incubated in the 37 °C DPBS, the constructs solely printed using Kca2 or Gel8 were dissolved within 1 hour. In contrast, the alternatively printed Kca2-Gel8 construct could maintain the 3D structure in the DPBS at 37 °C above 1 hour but not exceeding 1 day, which exhibited a better structural integrity than the Kca2 or Gel8 construct. Meanwhile, the structural integrity for the Kca2-Gel8 constructs in DI water and in DPBS, could last 5 days (see Figure 5.11) and < 1day (see Figure 5.12), respectively. The difference should be attributed to the difference in pH between DI water (pH=6.4) and the cell culture

medium (pH=7.0-7.4). For example, according to the supplier, the isoelectric point of Gel is 7-9. Thus, Gel exhibits positive charge when pH of DI water is below the isoelectric point, but shows negative charge when the pH is above the isoelectric point.

Figure 5.12 Images for the structural integrity of printed constructs in DPBS at 37 °C for different times.

GelMA is commonly used for bioprinting, where a stable 3D structure can be

formed by exposing the hydrogel to UV light to covalently crosslink the GelMA chains [90]. The GelMA construct solely printed using GelMA could keep their structural integrity in DPBS up to 30 days (see Figure 5.12). Additionally, the printed Kca2-GelMA10 construct could maintain its structural integrity above 20 days in DPBS. The traditional approach for printing GelMA is to utilize UV curing after printing each GelMA layer, which leads to a result that the first layer has been cured for many times before obtaining a 3D construct. It was reported that UV has potential harm to cells [4, 42]. In our study, the final construct was exposed to UV light for one time only after printing. This method can minimize harm to the cells due to UV exposure, especially to those cells printed in the first few layers.

On the basis of the structural integrity of Kca2-Gel8 and Kca2-GelMA10 constructs in DPBS at 37 °C, Kca2 and GelMA10 were known as the best combination to form a multilayered construct by printing them alternately to result in the best fidelity and structural integrity in DPBS. Additionally, the blend Kca2/GelMA10 was also prepared for printing. However, the blend cannot form a structure with a good shape fidelity. There is an obvious difference between the Kca2-GelMA10 construct and the Kca2/GelMA10 blend construct. In the blend of Kca2 and GelMA10, the positively charged ions and the negatively charged ions are homogeneously distributed. However, in the 3D printed construct, the electrostatic interaction occurs only at the surface between two oppositely charged hydrogels. Thus, the charge density at the interface between two oppositely charged hydrogels is much higher than

that in a blend. Moreover, the cast blend sample showed a poor structure integrity (see Figure 5.13) in DPBS at 37 °C. On the basis of the above founding, the blend was not used for further study.

Figure 5.13 Structural integrity of cast Kca2/GelMA10 sample in DPBS at 37 °C for different times

5.3.6 Cell viability in Kca2-GelMA10 construct

The representative fluorescent images of the bioprinted Kca2-GelMA10 constructs showed the live (stained green), dead (stained red), and the merged cells at day 0 and day 2, as shown in Figure 5.14 (a). Figure 5.14 (b) shows that all the tested samples exhibited a cell viability of more than 96%, which is comparable to that of the TCPS control. The cell viability in the Kca2-GelMA10 constructs at day 5 was also investigated. The cells were highly spreading, and forming the 3D network, as shown in Figure 5.14 (c). Thus, it was not easy to quantify the number of cells. However, this result indicates that the Kca2-GelMA10 construct has an excellent biocompatibility for cell proliferation. Moreover, the C2C12 morphologies in the printed Kca2-GelMA10 construct are shown in Figure 5.14 (d). At day 0, the C2C12 cells were rounded in the printed Kca2-GelMA10 construct. At day 5, almost all the

cells became elongated and highly spreading. The elongating and spreading morphology of cells can also be observed from Figure 5.14 (c).

Figure 5.14 (a) Live/dead staining of the C2C12 cells on bioprinted Kca2-GelMA10 constructs for day 0 and day 2. (b) Cell viability of C2C12 on TCPS control and the bioprinted Kca2-GelMA10 construct. (c) Live/dead staining of cells for the bioprinted construct at day 5. (d) OM images for the C2C12 cell morphologies on the bioprinted construct. Rounded cells are highlighted using arrows at day 0; at day 5, cells are highly spreading, as shown in the zoom-in image with an orange frame.

5.4 Summary

In this chapter, a promising strategy was reported to print a 3D construct with super strong interfacial bonding by utilizing the interactions between two oppositely charged hydrogels. Three anionic hydrogels (Alg, Xan, and Kca) and three cationic hydrogels (Chi, Gel, and GelMA) were chosen as the representatives of anionic and cationic hydrogels, respectively. The rheological properties of single hydrogels were investigated to simulate their behaviors before, during, and after printing. The printability of hydrogels, including shape fidelity and structural integrity in a cell culture medium, were examined as functions of the bioink concentration and combination. Finally, Kca2 and GelMA10 were found to be the best two oppositely charged hydrogels for successful printing of 3D constructs. The interfacial bonding strength between a Kca layer and a GelMA layer was proved to be significantly higher, compared to the bilayered Kca or bilayered GelMA. The bioprinted Kca-GelMA construct demonstrated an excellent biocompatibility with a cell viability of >96% up to day 2. Moreover, C2C12 elongated and built their own 3D network after culturing for 5 days. Based on the above findings, this novel method will open a new door for 3D bioprinting of layered constructs with a super strong interface bonding.

Chapter 6 Conclusions and Future Work

In this chapter, the main conclusions are drawn from the present research work, and then several recommendations are proposed for the future work.

6.1 Conclusions

The goal of this thesis was to select suitable hydrogels to successfully print 3D constructs for biomedical applications with the necessary considerations. Among all the considerations regarding the important properties of a candidate hydrogel and its generated 3D construct, this thesis focuses on: i) evaluating the printability of a candidate hydrogel from a rheological point of view; and ii) improving the interfacial bonding of a layered structure. Towards this end, a rheological approach for simulating the rheological behaviors of a hydrogel during the 3D printing process and estimating the printability of a candidate hydrogel was successfully presented; the strategies to print hydrogels constructs with strong interface bonding were successfully developed.

- Rheology is highly relevant to an extrusion-based 3D bioprinting process.

From a rheological point of view, this thesis clearly demonstrated the effect of 3D printing process on the rheological behavior of a candidate hydrogel and the resultant quality of printing for a 3D structure. Firstly, the shear rate generated during an extrusion process was estimated. After that, the rheological measurement at the estimated shear rate simulating the extrusion

process for 3D printing was conducted. The observed rheological properties (e.g., percentage of recovered viscosity, recovery time) of the tested hydrogel reflected the printability of the hydrogel.

- Interfacial bonding strength of a layered constructs could be improved through our developed strategies, including printing TSC at the interface with a post-crosslinking process, or exploiting the interaction between two oppositely charged hydrogels. After improving the interfacial bonding strength between two printed layers of a construct, the stackability of the hydrogel could be enhanced. For example, one of our strategies was to alternately print gelatin and a negatively charged hydrogel, which allowed gelatin to be not only a good bioink medium to load cells but also as a medium to provide positive ions to create the reaction with the negatively charged hydrogel (e.g. Kca) to improve the interfacial bonding of a multilayered structure. As an exciting result from improving the interfacial strength, the printed structure can stand by itself in liquid media (as compared to pure gelatin hydrogel) without further vigorous curing.

6.2 Future work

The learnings achieved in this thesis are that 3D hydrogel constructs with a good shape fidelity and strong interfacial bonding could be successfully fabricated with the assistance of the rheological selection of a candidate hydrogel and the interfacial

consideration of the printed 3D construct. Several future research directions are recommended as follows:

- It would be interesting to alternately print the Alg/MC blend hydrogel and the GelMA hydrogel. In the present work, the Alg/MC blend exhibits an excellent shape fidelity and stackability, while the GelMA hydrogel provides the cationic ions. As a bioink medium for loading cells for bioprinting, GelMA contains the peptide RGD, which promotes cell adhesion. This advantage makes GelMA is an appropriate medium for loading cells when compared to a TSC solution. Furthermore, the reaction between an anionic hydrogel and a cationic hydrogel could form super strong interfacial bonding. It is expected that delicate and thick constructs with strong interfacial bonding and good biocompatibility could be obtained through printing Alg/MC and GelMA alternately.
- The mechanical properties of the printed constructs during the culturing time at 37 °C are not examined in this thesis. These properties might change with time and could further affect the mechanical strength and the structure integrity of the printed structures. To give a better prediction, more mechanical measurements need to be done in the future.
- The working temperature for the bioprinter utilized in our study is around 26 °C. This temperature depends on the surrounding temperature on that day. Thus, future work can be carried out to improve the printing quality of a

hydrogel through adding the accessories to adjust the working temperature of the bioprinter, which could significantly affect the printing quality of a temperature sensitive hydrogel.

- Due to the limitation of the 3D printer used in this study (i.e. the highest pressure for printing is about 5 bar), the pressure is not sufficient to print hydrogels with a relatively high viscosity (such as Alg9/MC9). The highly viscous hydrogels might be extruded out through a nozzle when using the currently available highest pressure. However, a corresponding lower printing speed or a bigger nozzle should be used. Hence, to quickly obtain a construct with a higher resolution, the improvement of the 3D printing equipment is needed.

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